



2015

Tcr Modifications to Enhance Expression, Chain Pairing, and Antigen Recognition for Adoptive T Cell Transfer

Kendra Foley
Loyola University Chicago

Recommended Citation

Foley, Kendra, "Tcr Modifications to Enhance Expression, Chain Pairing, and Antigen Recognition for Adoptive T Cell Transfer" (2015). *Master's Theses*. Paper 2784.
http://ecommons.luc.edu/luc_theses/2784

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master's Theses by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.



This work is licensed under a [Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License](https://creativecommons.org/licenses/by-nc-nd/3.0/).
Copyright © 2015 Kendra Foley

LOYOLA UNIVERSITY CHICAGO

T CELL RECEPTOR MODIFICATIONS TO ENHANCE
EXPRESSION, CHAIN PAIRING, AND ANTIGEN
RECOGNITION IN T CELLS FOR ADOPTIVE T CELL TRANSFER

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

KENDRA C. FOLEY

CHICAGO, IL

MAY 2015

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. Michael Nishimura. I am very fortunate to have been given the opportunity to complete my thesis in his lab and under his mentorship. All of the incredible work he does over the years has been an inspiration and has motivated me to become the best scientist I can possibly be. I would like to thank him for all the help and guidance he has provided me with throughout my thesis project. Dr. Nishimura will continue to be a role model as I pursue my career in research.

I would also like to thank the members of my thesis committee, Dr. Makio Iwashima and Dr. Phong Le, for their guidance and suggestions throughout my project. Their expertise in the field and passion for research has been an inspiration for me.

I could not have completed this thesis without all the members of the Nishimura lab. I am grateful for the comradery we have in the lab that helped make working on my thesis project very enjoyable. I would like to thank them for all the help and support they have given me.

I would also like to thank the Department of Microbiology and Immunology. I am very appreciative for the opportunities I have received that helped me excel in both in my research and in my academic learning.

Statistical analysis was kindly provided by Elizabeth Garrett-Mayer. I would like to thank her for all the helpful insight she has given me.

I would also like to thank Patricia Simms and Ashley Hess for all their assistance in the FACS Core and helping me with cell sorting.

Lastly, I would like to thank my family and friends for their continuous love and support. I would especially like to thank my parents for their motivation and support throughout all of my academic years which has helped in achieving my goals and becoming the successful scientist I aspire to be.

Funding for these studies was provided by NIH/NCI Grant PO1 CA154778.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	xii
ABSTRACT	xiii
CHAPTER ONE: REVIEW OF LITERATURE	1
Introduction	1
T Cell Diversity	4
T Cell Development	4
T Cell Function	6
Adoptive T Cell Therapy	8
Modified T Cell Receptors to Enhance Chain Pairing	11
Concluding Remarks	14
CHAPTER TWO: MATERIALS AND METHODS	16
Cell Lines, Media, and Reagents	16
T Cells	17
Peptides	18
T Cell Receptor Modifications	18
Retroviral Vector Construction	19
Generating High Titer Producer Cell Lines	22
Retroviral PBMC Transduction	24
Immunofluorescence Staining	25
Cytokine Release Assay	25
CHAPTER THREE: RESULTS	27
Modified T Cell Receptor Design	27
Constructing Retroviral Vector Encoding Modified T Cell Receptors	30
Generating PG13 Producer Cell Lines	35
PBMC Transductions	37
Modified T Cell Receptor Function	49
CHAPTER FOUR: DISCUSSION	62
Modified T Cell Receptors to Enhance Proper Chain Pairing	62
Modified T Cell Receptor Cell Surface Expression	63
Modified T Cell Receptor Transduced T Cell Function	67

Concluding Remarks	70
APPENDIX A	72
Additional T Cell Function Figures	73
REFERENCES	89
VITA	92

LIST OF TABLES

Table	Page
1. Percentages of transduced T cells that express properly paired HCV 1406 TCRs	43

LIST OF FIGURES

Figure	Page
1. Potential TCR mispairing	3
2. Schematic representation of TCR gene modified T cells in adoptive T cell transfer	9
3. Strategy of vector subcloning and ligations	21
4. Generating high titer PG13 producer cell lines using HEK293GP packaging cells line	23
5. Structure of modified TCR constructs designed to prevent mispairing	29
6. Structure of the retroviral vector used for TCR gene transfer	31
7. Restriction enzyme digestion analysis of recombinant pCR2.1 clones	33
8. Restriction enzyme digestion analysis of recombinant SAMEN clones	34
9. CD34 surface expression on PG13 producer cells post CD34 purification	36
10. Donor 1 retrovirally transduced T cell populations	38
11. Donor 1 transduced T cell populations post CD34 magnetic bead sort	39
12. Cell surface phenotype of T cells transduced with T cells transduced with wild type HCV 1406 TCR or each modified HCV 1406 TCR	41
13. Comparison of donors among transduced reactive T cells transduced with wild type HCV 1406 TCR or modified HCV 1406 TCR.	46
14. Comparison of transduced reactive T cells transduced with wild type HCV 1406 TCR or modified HCV 1406 TCR.	48
15. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	51
16. Comparison of HepG2:NS3 T cell reactivity normalized to percent of	

tumor reactive T cells	53
17. Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	55
18. Log fold changes in peptide reactivity between T cells transduced with wild type TCR and T cells transduced with modified TCRs.	57
19. Log fold changes in tumor reactivity between T cells transduced with wild type TCR and T cells transduced with modified TCRs.	59
20. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	73
21. Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	74
22. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	75
23. Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	76
24. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	77
25. Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	78
26. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	79
27. Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	80
28. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	81
29. Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	82
30. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	83

31.	Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	84
32.	Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	85
33.	Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	86
34.	Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR	87
35.	Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells	88

LIST OF ABBREVIATIONS

°C	Degrees Celsius
BSA	Bovine serum albumin
C	Constant
CAR	Chimeric antigen receptor
CMV	Cytomegalovirus
DMEM	Dulbecco's Modified Eagle's Medium
D	Diversity
DN	Double negative
DNA	Deoxyribose nucleic acid
DP	Double Positive
ELISA	Enzyme-linked immunosorbant assay
EMEM	Eagle's Minimum Essential Medium
ER	Endoplasmic reticulum
HCV	Hepatitis C virus
HCV 1406	T cell receptor recognizing the Hepatitis C virus protein NS3 1406-1415 amino acid sequence
HLA	Human leukocyte antigen
IFN- γ	Interferon- γ
ITAM	Immunoreceptor tyrosine-based activation motif

J	Joining
Kb	kilobase, one thousand base pairs
LB	Luria-Bertani
LTR	Long terminal repeats
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mL	Milliliter
Murinized	Of mouse origin
ng	Nanogram
NHEJ	Nonhomologous end joining
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
RS	Recombination signal
RT	Room temperature
SA	Splice acceptor
SD	Splice donor
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TIL	Tumor infiltrating lymphocyte
Tyro	Tyrosinase
μL	Microliter

V	Variable
VSV	Vesicular stomatitis virus

ABSTRACT

T cell receptor (TCR) gene modified T cells for adoptive T cell transfer therapy have been shown to mediate clinical success in treating melanoma and other malignancies by redirecting the specificity of peripheral blood lymphocytes (PBL) to recognize tumor and/or viral associated antigens of choice. One of the challenges in using TCR gene modified T cells for adoptive transfer include proper expression and function of the introduced TCR. Mispairing between endogenous and introduced alpha and beta TCR chains allows for the potential of unanticipated off-target reactivity or autoimmunity. Additionally, chain mispairing reduces expression of the introduced TCR which can result in impaired therapeutic efficacy against targeted antigens. One approach to improve TCR expression and pairing on the cell surface and to enhance T cell function involves the modification of the introduced TCR genes to promote proper pairing. Some of the modifications designed to augment proper TCR pairing include introducing a disulfide bridge in the alpha/beta constant regions, substituting human with murine constant regions, codon optimization to enhance protein synthesis, TCR chain leucine zipper fusions, and a single chain TCR.

We have previously identified and cloned a novel TCR from an HLA-A2 restricted, HCV NS3:1406-1415-reactive T cell clone and successfully redirected antigen specificity of normal PBL-derived T cells using a recombinant retroviral

vector encoding TCR genes. We have also developed a unique surface transduction marker, CD34t, that allows for the exact measurement of TCR translation due to the 1:1 stoichiometric ratio between CD34t and the TCR proteins. This allows us to directly compare the TCR expression and T cell function of each TCR modification using this internal reference standard.

Our results revealed that the murinized C β 2 TCR and the leucine zipper TCR have the highest levels of cell surface expression per transduced T cells when compared to the wild type TCR. It is also evident in this study that although some modifications have higher levels of TCR cell surface expression, this does not always result in increased T cell function. Our studies have given us a better understanding of how these TCR modifications can impact TCR expression and T cell function that may allow for optimization of TCR modified T cells for adoptive cell transfer to treat patients with viral infections and malignancies.

CHAPTER ONE

REVIEW OF LITERATURE

Introduction

T cells play a vital role in adaptive immunity in fighting both microbial and viral infections as well as malignancies. The specificity of this immunity lies within the T cell receptor (TCR). Therefore, understanding and utilizing the TCR mechanisms such as TCR antigen recognition and T cell activation involved in the adaptive immune response is critical for the success of different treatments or therapies.

Although the adaptive immune response is capable of recognizing infections and malignancies, the response is not always efficient enough for clearance. Adoptive T cell transfer therapy has been shown to have clinical success in treating melanoma, other malignancies, and viral infections. This process involves isolating tumor infiltrating lymphocytes (TILs), expanding them *ex vivo*, and subsequent administration back into the patient [1]. The expansion of these cells can be difficult and is not always possible with a wide variety of cancers [2]. An alternate strategy that has been clinically successful is the use of genetically modified T cells for adoptive cell transfer. The transduction of T cells or NK cells with chimeric antigen receptors (CARs) or TCRs has been shown to redirect the specificity of these cells to recognize tumor and/or viral associated

antigens [3]. By isolating tumor reactive T cell clones from TIL or peripheral blood lymphocyte derived T cells and identifying and cloning the TCR genes that facilitate recognition of tumor associated antigens or viral antigens, it is possible to generate a library of these reactive TCRs. In this thesis we will exclusively study TCR biology by using a retroviral vector encoding TCR genes, to redirect the specificity of T cells to recognize specific tumor or viral antigens.

TCR gene modified T cells for adoptive T cell transfer is a form of more personalized medicine, since its procedure is unique for each individual patient. Because personalized medicine is becoming more popular and the methodologies used in this therapy can be applied to a variety of cancers and viral infections, it is critical to focus on making this therapy as clinically efficient and effective as possible. One of the major challenges with using TCR gene modified T cells for this type of therapy is the potential of alpha and beta chain mispairing between endogenous and introduced TCRs. A representation of this is shown in Figure 1. This not only reduces the proficiency of the T cell but also allows for the potential of self-antigen recognition which could lead to autoimmunity. One strategy to improve this form of therapy is to modify the TCR proteins in a way that promotes proper pairing of the introduced TCRs, resulting in a higher level of expression, and increased functionality to make a better T cell. This strategy will be explored in more detail in this thesis.

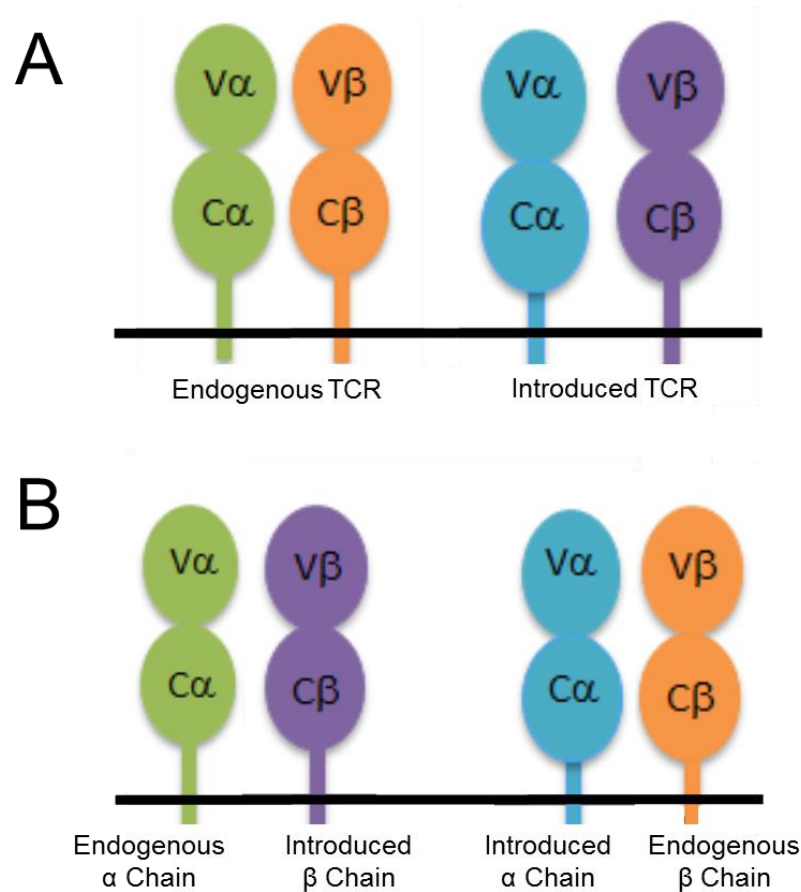


Figure 1. Potential TCR mispairing.

One of the potential consequences of introducing another TCR in a T cell is the potential alpha and beta chain mispairing between the endogenous TCR and the introduced TCR. The ideal outcome is shown in panel A. The endogenous alpha chain (green) is properly paired with the endogenous beta chain (orange) and the introduced alpha chain (blue) is properly paired with the introduced beta chain (purple). The potential mispairing is shown in panel B. The endogenous alpha chain (green) could pair with the introduced beta chain (purple) or the introduced alpha chain (blue) could pair with the endogenous beta chain (orange).

T Cell Diversity

Since this thesis focuses on TCR gene modified T cells, we will focus only on T cell biology and genetics. The vast repertoire of TCRs is a product of somatic gene rearrangement of the TCR gene loci and allows for recognition of roughly 10^{18} epitopes [4]. The variable (V), diversity (D), joining (J), and constant (C) regions make up the TCR gene segments. V(D)J recombination occurs in the thymus and is essential for the generation of diverse TCRs. This recombination is site-specific and occurs only between TCR gene segments that are flanked by recombination signal (RS) sequences [4]. Recombination activating genes-1 and -2 (RAG-1 and RAG-2) bind to the RSs and make single strand nicks in the DNA to initiate recombination. First, the TCR beta chain D-J gene rearrangements occur, followed by V-DJ gene rearrangements. Nonhomologous DNA end-joining (NHEJ) proteins join the rearranged gene fragments [4]. This recombination is responsible for the large diversity in TCRs that allows for the recognitions of so many pathogens.

T Cell Development

T cells develop from hematopoietic stem cell progenitors derived from the bone marrow that travel to the thymus for T cell development. At this point, these cells are TCR⁻, CD4⁻, and CD8⁻ and are termed double negative (DN). There are 4 differentiation stages which are determined by various markers on the cell surface. During stages DN2 through DN4 the developing T cell expresses a pre-TCR [5]. This pre-TCR consists of a fully rearranged TCR beta chain and a non-

rearranged pre-TCR alpha chain in association with the CD3 signaling complex in order to assure active and functional signaling. The formation of this pre-TCR has been shown to be critical for T cell maturation and also rescuing the developing T cell from programmed cell death. It functions to control allelic exclusion of the TCR beta locus as well as permitting TCR alpha chain rearrangement [6]. With a fully rearranged TCR alpha beta on the surface, the T cell becomes double positive (DP), expressing both CD4 and CD8 co-receptors [5]. These T cells can move onto the next step in T cell development.

The next step in T cell development involves positive and negative selection of the T cells and occurs in the thymic cortex. T cells interact with peptide-MHC complexes on stromal or dendritic cells via their TCR. There are a few different possibilities that could occur as a result of this interaction. If there is no recognition of the TCR with MHC and self antigen, death by neglect will occur. If the TCR affinity is too strong, the T cell is negatively selected and apoptosis occurs [5]. Negative selection is to avoid any potential chance of autoimmunity. The TCR must recognize self antigen in the context of MHC expressed on the antigen presenting cell with a weak interaction to be positively selected [5]. Upon positive selection, the T cells migrate to the medulla and then becoming single positive, expressing either CD4 or CD8 co-receptor, depending on the initial TCR signal [7]. When activated, these T cells can now migrate to the periphery to elicit an immune response.

T Cell Function

Activation of the T cell is dependent on the engagement of the TCR with peptide in context of either MHC Class I or MHC Class II. Both MHC class I and MHC class II have three genes encoding the MHC molecules within the HLA complex in humans. For the classical genes, these include HLA-A, -B, and -C for MHC class I and HLA-DR, -DQ, and -DP for MHC class II [8]. These genes are highly polymorphic which allows for a wide genetic variation within the peptide binding groove [9]. The peptide binding platforms of the MHC molecules in which the peptide is positioned are different.

The peptides that are presented on the MHC are the products of two major proteolytic mechanisms [8]. In MHC class I peptide binding, proteins in the cytoplasm get degraded by a proteasome and are then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing protein (TAP). Peptides bind to the MHC class I in the peptide loading complex and are then transported through the golgi to the cell surface and can be recognized by CD8⁺ T cells [8].

In contrast, in MHC class II peptide binding exogenous antigen from outside the cell gets endocytosed and is delivered to late endosomes. In endosomes, the antigen gets processed by cathepsins. At this point, MHC class II protein and the chaperone HLA-DM protein have already passed through the golgi into the late endosome. Peptides are loaded onto the MHC by HLA-DM and

are then transported to the cell surface and can be recognized by CD4⁺ T cells [8].

CD4 and CD8 glycoproteins are important for T cell activation. They act by stabilizing the physical interaction between the T cell and antigen presenting cell via the MHC on the antigen presenting cell [10]. In addition to enhancing stability, it has been shown that the cytoplasmic tails of CD4 and CD8 aid in co-receptor function by binding to Lck for translocation to the CD3 signaling complex [11]. During T cell activation due to TCR engagement, the Src-family kinase Lck will phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMS) on the CD3 ζ chain. Studies have shown that CD4 and CD8 enable the recruitment of these Lck proteins that are necessary to initiate T cell signaling [12]. CD4 and CD8 are shown to be important co-receptors due to their dual functions.

TCR signaling in response to antigen recognition is essential for a robust immune response. In addition to the TCR, the CD3 signaling complex is of critical importance for both TCR expression and function. The CD3 complex consists of ϵ , γ , δ , and ζ subunits. These subunits form a CD3 $\epsilon\gamma$ heterodimer, a CD3 $\epsilon\delta$ heterodimer, and a CD3 $\zeta\zeta$ homodimer [13]. The ζ chain contains ITAMs. Upon antigen recognition by the TCR, the ITAMs become phosphorylated by the Src-family kinase Lck [14]. Zap-70 is then recruited to these phosphorylated ITAMS, is phosphorylated by itself, and induces a cascade of downstream signaling pathways [15]. Through multiple signaling pathways, there are profound changes in gene expression leading to the production of cytokines. A majority of these

changes are facilitated by the transcription factors NFAT, NF- κ B, and AP1.

Cytoskeletal remodeling occurs in addition to the vast increase in cytokine production [14]. Understanding the way a T cell becomes activated and functions via TCR engagement is important for application in terms of TCR gene modified T cells.

Adoptive Cell Therapy

The observation of TILs present in the tumor lesions has eventually led to the idea of using T cells to target cancer. *Ex vivo* TIL expansion and cell transfer to melanoma patients resulted in responses in about 34% of patients [16].

Although this can be an effective treatment, TIL can be difficult to isolate and expand from both melanoma and other cancers [2]. An alternative approach in targeting tumor associated antigens is genetically modifying a patients T cells with an antigen specific TCR. The idea of this therapy is to use TCR gene modified T cells for adoptive T cell transfer. In addition to melanoma and other cancers, this approach could also be used to treat viral infections. The process of this therapy is shown in Figure 2.

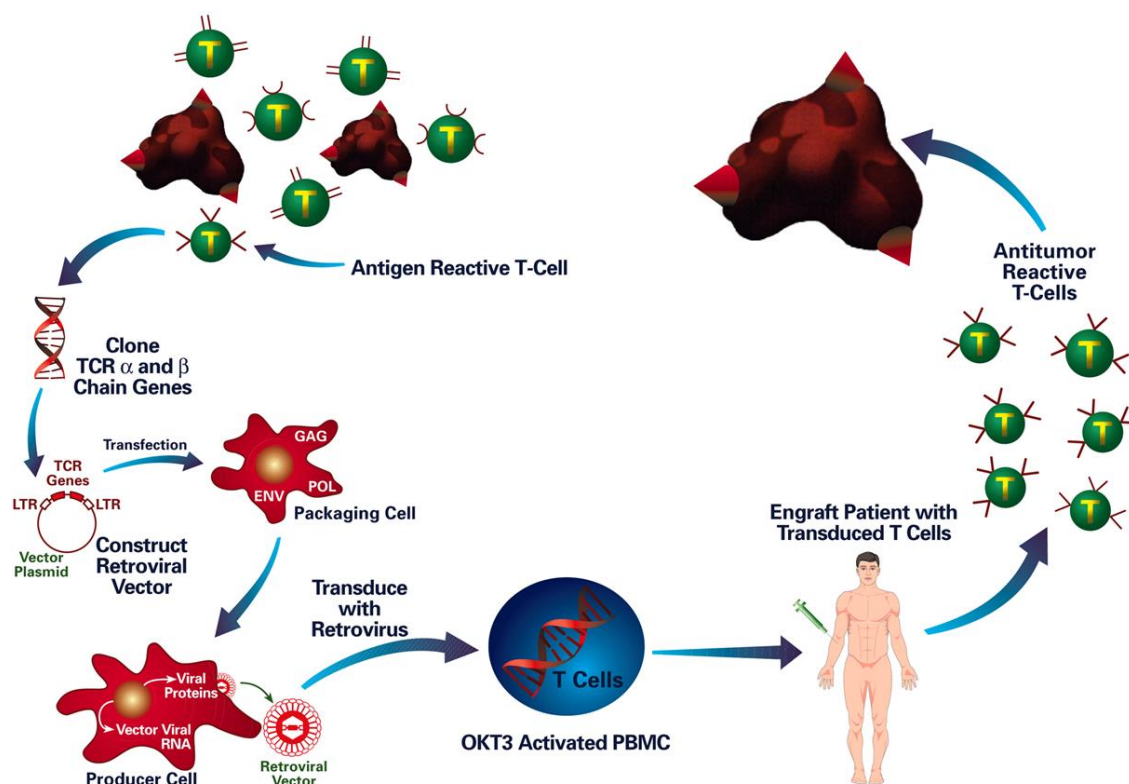


Figure 2. Schematic representation of TCR gene modified T cells in adoptive T cell transfer.

Tumor reactive T cell clones are isolated and expanded from the peripheral blood mononuclear cells (PBMC) or TIL of a patient with cancer. TCR genes that are reactive against tumor associated antigens or viral antigens are identified and cloned into a retroviral vector. Packaging and producer cell lines are utilized to make high titer retrovirus. Patient OKT3 activated peripheral blood T cells are transduced with retroviral supernatant, expanded, and administered back to the patient. The specificity of these circulating T cells is now redirected toward a specific tumor or viral antigen resulting in anti-tumor immunity.

Using TCR gene modified T cells in adoptive T cell transfer therapy has been shown to have clinical success by redirecting the specificity of PBLs to recognize tumor and/or viral associated antigens of choice. One of the strategies developed in the Nishimura lab uses retroviral vectors encoding TCR genes to redirect the specificity of normal peripheral blood lymphocytes (PBL)-derived T cells to recognize tumor and viral antigens. We have previously identified and cloned a novel TCR from an HLA-A2 restricted, Hepatitis C Virus (HCV) NS3:1406-1415-reactive CD8⁺ T cell clone [17]. It has been determined that this TCR is reactive in the absence of CD8. Therefore, due to its high affinity, in contrast to other TCRs, this TCR can overcome the need for CD8 co-receptor expression [18]. This is advantageous first, to generate MHC class I-restricted CD4⁺ T cells and secondly, lower transgene expression is needed to activate the transduced T cells [3]. Because of the advantages stated above, this HCV TCR is an excellent TCR to use in TCR gene modified T cells for therapy.

Using TCR gene modified T cells in adoptive T cell transfer is an attractive therapy for the treatment of malignancies and viral infections. Despite this, there are still hurdles in achieving an effective therapy. Some of these include proper TCR expression and function due to mispairing of alpha and beta chains between endogenous and introduced TCRs, and proper folding and assembly on the cell surface. Reduced cell surface expression of the TCR and reduced T cell functionality can result in impaired therapeutic efficacy against targeted antigens. The chain mispairing can allow for the potential of unanticipated off-target

reactivity or autoimmunity since these TCRs have not been subjected to the process of negative selection [19]. Although never seen in humans, it has been shown in mouse models that the formation of self-antigen reactive TCR dimers can result in TCR gene transfer-induced graft versus-host disease [20]. My study focuses on a strategy to address this potential TCR chain mispairing.

Modified T Cell Receptors to Enhance Chain Pairing

Introducing different modifications to the introduced TCR genes is one strategy to improve TCR expression on the cell surface, chain pairing, and T cell function. Various modifications have been done to directly and indirectly augment proper pairing of introduced TCR chains.

One approach is the addition of another disulfide bond in the TCR chains. Studies have shown engineering point mutations in each alpha and beta TCR chain constant region will favor the formation of an additional disulfide bond. Data revealed not only an increase of properly paired TCRs on the surface but also an increase in the total amount of introduced TCR when compared to the wild type TCR [21]. It was hypothesized that these TCRs with cysteine modifications could be less capable of mispairing and forming mixed dimers due to abnormal folding [21]. Enhanced IFN- γ production has also been shown in comparison to wild type when cocultured with TCR respective melanoma cell lines [22]. This approach has been shown to be an effective strategy in lowering the potential of TCR mispairing.

A second TCR modification involves replacing human constant regions with murine constant regions. When murine constant regions were placed in human TCRs, the TCRs had a higher surface expression as well as cytokine production in comparison to the wild type human constant region TCR [23]. It was also observed that the mouse constant regions outcompete human constant regions for CD3 which allows for a better association and higher affinity for the CD3 ζ chain [23]. One concern with a murinized TCR is that there is a potential risk of increased immunogenicity [24]. Substitution of just nine murine amino acids have been shown to be sufficient enough to allow for a higher surface expression and function of the TCR [24]. In order to lower the chance of immunogenicity, it could be beneficial to only use these substitutions instead of the whole murine constant region [24]. Based on these findings, murinized TCR constant regions in place of human TCR constant regions appears to be a successful approach in promoting proper pairing.

Codon optimization of the transgenic TCR gene has been shown to be another successful strategy for increased surface expression and function. Unlike the other TCR modifications, a codon-modified TCR does not directly aid in proper TCR chain pairing yet still had a higher level of expression when compared to the wild type TCR [25]. This is a result of enhanced mRNA translation that allows for increased amounts of protein synthesis [25]. Therefore by increasing the protein production of this TCR, there is a better chance for it to correctly pair and outcompete the endogenous TCR. Codon optimization is

strategy shown to be successful in increasing TCR surface rather than promoting proper TCR chain pairing.

A fourth TCR modification involves using a leucine zipper fusion protein. It has been shown that heterodimerization domains of the c-Jun and v-Fos transcription factors can be fused to the C-terminus of TCR alpha and beta chains via a short linker [26]. These proteins will then assemble to form a stable heterodimeric coiled coil that was termed a leucine zipper [27]. The leucine zippers that could potentially be formed into a homodimer are unstable and therefore there is a thermodynamic driving force in favor of the heterodimer formation [28]. Based on these studies, this leucine zipper TCR modification is shown to be an effective way to promote proper TCR chain pairing.

Lastly, a single chain TCR is another modification that has been shown to have success in preventing mispairing. The structure of this TCR links the variable alpha domain to the variable beta domain followed by the constant beta domain [29]. The constant alpha domain is made as a single protein. This alpha constant region is shown to hardly pair with an endogenous beta chain mostly likely because the interchain affinity is too weak to allow pairing [30]. This TCR modification appears to be a successful approach in promoting proper TCR chain pairing.

While all of the TCR modifications explained above have been shown to be successful strategies in promoting proper TCR chain pairing, they have never been compared together in the same experiments. This study focuses on

comparing all these different modifications using our unique transduction marker, CD34t. We have developed this unique surface transduction marker, CD34t, that allows for the exact measurement of TCR protein expression because there are no limitations on its cell surface expression and consequently CD34t and the TCR proteins are translated in a 1:1 stoichiometric ratio. The truncated CD34 protein lacks the signaling domain and therefore will not induce cellular activation when bound to antibody [19]. Using this CD34t transduction marker, we can compare all of the TCR modifications based on this internal reference standard.

Concluding Remarks

Using TCR gene modified T cells in adoptive T cell transfer is an attractive therapy for treating malignancies and/ or viral infections. In order to optimize the immune response in this novel therapy, there are still a few challenges to be addressed. One significant hurdle this study focuses on improving is the potential of mispairing between endogenous and introduced TCRs. By making six different modifications to the HCV 1406 wild type TCR, we can analyze the TCR cell surface expression and T cell function in comparison to the wild type TCR. Additionally, our unique CD34t transduction marker is beneficial in this study because it allows us to compare all of these TCRs with the same internal reference standard. We hypothesize that through the modifications of the retrovirally introduced TCR, more properly paired TCRs will be expressed on the cell surface, which will allow for better T cell function and anti-tumor immunity.

We will also be able to determine if there is a modification that appears to be superior to others and would therefore be beneficial to use in TCR gene modified T cells for adoptive T cell transfer therapy.

CHAPTER TWO

MATERIALS AND METHODS

Cell Lines, Media, and Reagents

T2, HEK293GP, PG13, and HepG2 cell lines were obtained from the American Type Culture Collection (Rockford, MD). T2 cells are TAP deficient and therefore cannot load their own peptide onto MHC class I. The MHC becomes stable on the cell surface when loaded with exogenous peptide. These T2 cells were used as stimulator cells for T cell functional assays. HEK293GPs are a human embryonic kidney packaging cell line that were made to express the retroviral gag and polymerase proteins. HEK293GP cells are used to produce high titer retrovirus by transient co-transfection using a retroviral vector and a plasmid containing the vesicular stomatitis virus (VSV) envelope. PG13 cells are a retroviral producer cell line that when transduced with HEK293GP supernatant, will stably produce high titer retrovirus. HepG2 is a HLA-A2⁺ human liver carcinoma cell line and are used as stimulators in a coculture system. HepG2 tumor cell lines have been engineered to express the full length HCV NS3 protein and were kindly provided by Timothy Spear. The cell line was maintained in complete medium as described below.

All medium components were obtained from Corning Life Sciences, unless otherwise noted. T2 cells were maintained in RPMI 1640 medium supplemented

with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA).

HEK293GP cells were maintained in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum.

PG13 cells were maintained in complete medium consisting of Iscove's DMEM supplemented with 10% fetal bovine serum. HepG2 cells were maintained in complete medium containing Eagle's Minimum Essential Medium (EMEM) .

T Cells

All PBMC used in this study came from apheresis products purchased from Key Biologics (Memphis, TN). PBMCs were isolated from three independent normal healthy donors by Ficoll-Paque (GE, Fairfield, CT) density gradient centrifugation. Briefly, whole blood was diluted 1:2 in Phosphate Buffer Saline (PBS) (Mediatech, Inc., Manassas, VA), loaded onto Ficoll density gradients, and spun at 2,000 RPM for 30 minutes. The density of Ficoll is 1.077 g/mL which allowed red bloods to pass through the Ficoll and inhibited the passage of white blood cells. Buffy coat was collected and washed three times with PBS.

All T cells were maintained in complete T cell medium consisting of AIM V medium (Life Technologies) supplemented with 5% heat-inactivated pooled human AB serum (Valley Biomedical, Inc.), 300 IU/mL recombinant IL-2 (rhIL-2; Novartis Pharmaceuticals Cooperation, East Hanover, NJ), and 100 ng/mL recombinant human IL-15 (rhIL-15; National Institutes of Health) at 37°C in a humidified 5% CO₂ incubator.

Peptides

HCV NS3:1406-1415 (KLVALGINAV) and Tyrosinase:368-376 (TMDGTMSQV) were obtained from Synthetic Biomolecules (San Diego, CA) and were HPLC purified at 95%. Peptides were stored at 5µg/mL in 100% DMSO at -80°C.

T Cell Receptor Modifications

Six modified TCRs were made using the wild type HCV TCR sequence. A disulfide bridge was introduced into the wild type TCR by making changes T178C and S721C in the constant region of the alpha and beta chains, respectively [31]. This was done by using a GENEART site directed mutagenesis kit (Invitrogen) in the SAMEN vector with the following primers (IDT, Coralville, IA), bases changes are denoted by bold and underline:

α forward: 5'-GTATATCACAGACAAA**TG**TGTGCTAGACATGAGG-3',

α reverse: 5'-CCTCATGTCTAGCAC**CA**TTTGTCTGTGATATAC-3',

β forward: 5'-GTGCACAGTGGGGTC**TG**CACAGACCCGCAGC-3',

β reverse: 5'-GCTGCGGGTCTGTGC**AG**ACCCCACTGTGCAC-3'.

All other HCV 1406 modified TCRs were synthesized by GenScript and provided in the pUC57 vector (GenScript, Piscataway, NJ). Two murinized TCRs were made by replacing human constant regions with murine constant regions. There are two murinized TCRs, Cβ1 and Cβ2, because there are two murine beta constant regions. These changes were designed base on sequences found

in GenBank. The wild type TCR was codon optimized by GenScript and provided in the pUC57 vector. In the leucine zipper TCR, the heterodimerization motif of the c-Fos or v-Jun was fused to the C-terminus of the alpha or beta chain respectively via a short linker as described [26]. In the single chain TCR, the two variable regions were linked via a 15-residue glycine linker to allow for correct folding. The three-domain TCR ($V\alpha$ - $V\beta$ - $C\beta$) was followed by a self-cleaving 2A sequence and the $C\alpha$ region [29].

Retroviral Vector Construction

Each of the modified TCRs were subcloned the same way from the pUC57 vector to the pCR2.1 vector containing C3D4t to the SAMEN vector was done and this process is shown in Figure 3. Briefly, vector DNA was transformed into *Escherichia coli* TOP10 competent cells onto LB Ampicillin plates (100 μ g/mL) and colonies were expanded in superbroth (32 grams Tryptone (Fisher), 20 grams yeast extract (Fisher), 5 grams NaCl (Fisher), 1 liter de-ionized water) with 100 μ g/mL Ampicillin (Sigma-Aldrich). Plasmid DNA from recombinant clones was isolated using a Miniprep kit (Qiagen) and screened for the presence of the TCR by restriction enzyme digest analysis. TCR genes were digested with NotI and EcoRI enzymes (Thermo Scientific) and products were separated on a 1% agarose gel. DNA bands corresponding to correct length of the TCR were excised from the gel and purified by a gel purification kit (Qiagen). The TCR genes were subcloned into the pCR2.1 TA cloning vector with compatible

restriction sites using a ratio of 1:5, vector DNA to insert DNA. The ligation reaction was incubated at 16°C overnight with T4 DNA Ligase and 10X T4 DNA Ligase Buffer (New England Biolabs, Ipswich, MA). The ligation product was transformed and DNA was isolated using a Miniprep kit. The TCR α -P2A-TCR β -T2A-CD34t fragment was then excised from pCR2.1 and cloned into the SAMEN vector with NotI and BAMHI restriction sites as described above. All HCV 1406 TCR constructs in SAMEN vector were sequenced (Genewiz, South Plainfield, NJ) to ensure no errors had occurred.

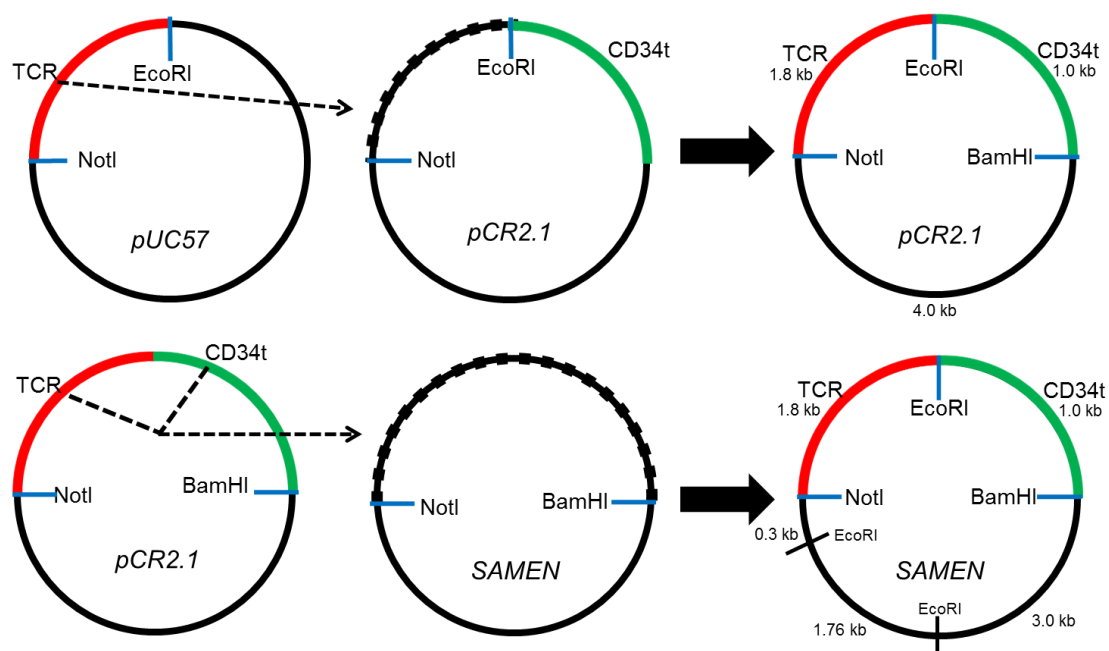


Figure 3. Strategy of vector subcloning and ligations.

Disulfide bridge, murinized, codon optimized, leucine zipper and single chain modified TCRs were excised from the *pUC57* vector using the restriction sites *NotI* and *EcoRI* and ligated into the *NotI* and *EcoRI* digested *pCR2.1* vector containing CD34. The resulting construct is shown above with the TCR alpha and beta chains fused to CD34t in the *pCR2.1* vector. The TCR and CD34t fragment was then excised from the *pCR2.1* vector using the restriction sites *NotI* and *BamHI* and ligated into the *NotI* and *BamHI* digested *SAMEN* vector. The resulting construct is shown above, is the *SAMEN* vector now containing the TCR genes and the CD34t gene.

Generating High Titer Producer Cell Lines

Using a HEK293GP packaging cell line, retroviral supernatants were prepared and used to make a stable retroviral producer cell line PG13 expressing HCV 1406 TCR in SAMEN vector as described [30]. A simplified flow chart of this process is shown in Figure 4. On day 0, HEK293GPs were seeded at 3 million, plated in 10 cm poly-D-Lysine coated plates (Corning) 100 µg/mL in 10 mL complete medium and incubated overnight at 37°C in 5% CO₂. HEK293GP cells were transiently co-transfected with 20 µg retroviral SAMEN vector DNA and 5 µg of a plasmid containing the VSV envelope gene using 50 µL Lipofectamine 2000 (Invitrogen) on day 1. Transfection medium was replaced 6 hours later with 10 mL fresh complete medium and incubated for 48 hours at 37°C in 5% CO₂. On day 2, PG13 cells were seeded at 2 million in a 10 cm tissue culture plate in 10 mL complete medium and incubated at 37°C in 5% CO₂. On day 3, fresh HEK293GP viral supernatant was collected and filtered to sterilize using a 0.45 µm filter to sterilize (Millex, Billerica, MA). PG13 media was replaced with filtered HEK293GP viral supernatant and 3 mL complete medium. Plates were incubated for 72 hours at 37°C in 5% CO₂. On day 6, cells were collected and stained using anti-CD34-PE mAb (BioLegend) and analyzed for CD34 expression by flow cytometry. Cells were stained with an anti-CD34-PE mAb and CD34 positive cells were sorted for high and uniform expression using a BD FACSAria cell sorter (BD BioSciences, San Jose, CA) and the final cells were maintained in complete medium.

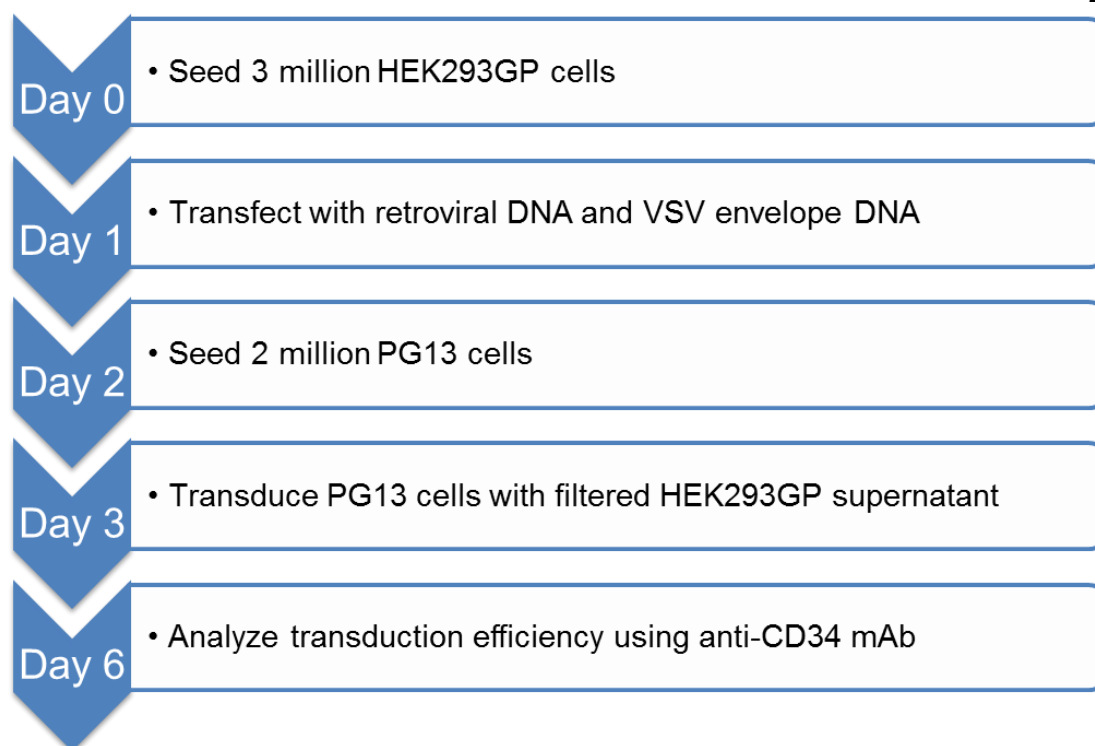


Figure 4. Generating high titer PG13 producer cell lines using HEK293GP packaging cell lines.

On day 0, 3 million HEK293GPs were seeded. On day 1, 20 µg of retroviral DNA and 5 µg of VSV envelope DNA were added to the HEK293GP plates. 6 hours later the media was replaced with fresh complete medium. On day 2, 2 million PG13 cells were seeded. On day 3, HEK293GP viral supernatant was filtered and plated onto the PG13 cells. On day 6, cells were collected and stained using an anti-CD34-PE mAb and analyzed for CD34 expression by flow cytometry. Depending on percent transduction, CD34 positive cells were sorted for high and uniform expression a few days later.

Retroviral PBMC Transduction

T cells derived from normal healthy donors were activated by adding 50 ng/mL anti-CD3 monoclonal antibody (Miltenyi Biotec, San Diego, CA), 300 IU/mL rhIL-2 (Proleukin, San Diego, CA), and 100 ng/mL rhIL-15 (NCI-Frederick, Frederick, MD) on day 0. To make high titer retroviral supernatant, HCV 1406 TCR expressing PG13 cell lines were seeded overnight at 8×10^6 cells/T-175 flask at 37°C in 5% CO₂ on day 1. On day 2, complete Iscove's DMEM supplemented with 1mM sodium butyrate (Sigma-Aldrich) and 10 mM HEPES was added to flasks for 8-10 hours to stimulate virus production. Media was then replaced with fresh complete medium and incubated overnight at 37°C in 5% CO₂. Fresh viral supernatants were collected on day 3 and filter sterilized and to remove any cellular debris using 0.45 µm filters (Thermo Scientific).

T cells were transduced by spinoculation on day 3 as described [16]. Briefly, 24-well-flat-bottom-non-treated tissue culture plates were coated with Retronectin (Takara, Mountain View, CA) overnight. The next day, plates were blocked using 2% BSA (bovine serum albumin) in PBS (Thermo Scientific) for 30 minutes. Next, virus was loaded by adding 2 mL of fresh retroviral supernatant per well and plates were spun for 2 hours at 2,000xg at 32°C. 1 mL of the activated T cells in complete medium were added to the plates at 2×10^6 million/mL along with 1 mL of fresh viral supernatant. The plates were spun again for 2 hours at 2,000xg at 32°C. After 24 hours, the transduced T cells were transferred to flasks and plated at 1×10^6 /mL. On day 7, transduction efficiency

was determined by FACS analysis using anti-CD34-PE mAb. Transduced T cells were purified by positive selection using CD34 magnetic beads (Miltenyi Biotec, San Diego, CA) and maintained in complete T cell medium. The T cells were used in functional assays beginning on day 13.

Immunofluorescence Staining

PG13 cells were stained for CD34 surface expression by immunofluorescence using anti-CD34-PE. This was done to confirm retroviral transductions were successful and to measure transduction efficiency. T cell surface markers were stained by immunofluorescence using the following mAbs: anti-CD4-PE/Cy7, anti-CD8-PerCP/Cy5.5, anti-CD3-APC/Cy7, and anti-CD34-PE (BioLegend, San Diego, CA). Surface expression of properly paired HCV 1406 TCRs were stained by using a APC-labeled HLA-A*0201 dextramer folded around HCV NS3:1406-1415 (Immudex, Fairfax, Virginia). This dextramer was used for this study because it can bind only properly paired TCRs. 1×10^6 cells were analyzed by using a BD FACSCanto II instrument (BD Biosciences, San Jose, CA) by collecting 50,000 events and data was analyzed by FlowJo software (FlowJo Enterprise, Ashland, OR).

Cytokine Release Assay

Antigen reactivity of the HCV 1406 TCR transduced T cells was measured in cytokine release assays as described [32]. Briefly, all peptide loaded T2

stimulators were pulsed with 10 µg/mL HCV 1406 peptide or Tyrosinase peptide for two hours prior to coculture. 1×10^5 washed and re-suspended responder T cells and stimulator cells (peptide loaded T2 cells or HepG2 tumor cells) were cocultured in a 1:1 ratio in 96-well U-bottom tissue culture plates in 200 µL complete medium. Cocultures were incubated at 37°C in 5% CO₂ for 20 hours. Plates were spun at 1,500 RPM and supernatants were collected for analysis of cytokine release.

The amount of cytokine released was measured via sandwich ELISA using monoclonal antibodies to IFN-γ (BioLegend, San Diego, CA). Briefly, 96-well plates were coated overnight at 4°C with hIFN-γ mAb. The next day, plates were washed with ELISA wash buffer (1X PBS, 0.01% Tween) then blocked with 200 µL 1X Assay Diluent (phosphate buffered saline solution containing bovine serum) for 2 hours at room temperature (RT) on a shaker. Plates were washed again with ELISA wash buffer and 100 µL of sample and standards were added. After 2 hours of shaking at RT, plates were washed and 100 µL of the enzyme-conjugated detection mAb was added. After 1 hour of shaking at RT, 100 µL of Avidin-HRP was added and incubated on the shaker for 30 minutes at RT. Plates were then washed and 100 µL of TMB Substrate Solution was added. Plates were incubated at RT in the dark for 20 minutes or until color developed in standard curve. 100µL of 2N H₂SO₄ was used to stop the reaction. The absorbance of the plates was read at 450 nm.

CHAPTER THREE

RESULTS

Modified T Cell Receptor Design

One of the challenges in using TCR modified T cells in adoptive T cell therapy is the potential of TCR chain mispairing between the endogenous and introduced alpha and beta chains (as shown in Figure 1). One of the strategies to promote proper pairing is to make modifications to the TCR genes. This study focuses on comparing six different TCR modifications to a wild type TCR by comparing both TCR cell surface expression and T cell functionality. This study is unique compared to other TCR pairing studies in that CD34t is used as a cell surface marker of transduction. CD34t and the TCR genes are translated in a 1:1 stoichiometric ratio which allows for the direct comparison of these TCR modifications based on the CD34t internal reference standard. The goal of this study is to determine if there is an optimal modification that demonstrates high levels of properly paired TCRs expressed on the cell surface as well as improved T cell functionality.

The first modification is the disulfide bridge TCR. By introducing two cysteine residues using site directed mutagenesis, an additional disulfide bond was constructed in the constant regions of the alpha and beta chains. This modification covalently links the two chains, therefore promoting proper pairing

and providing stability [21] . The second and third modifications consist of replacing the human alpha and beta chain constant regions with murine constant regions. Because there are two different murine beta constant regions ($C\beta_1$ and $C\beta_2$), we made two murinized TCRs. Since a murine constant region cannot pair with a human constant region, murinization promotes proper pairing and additionally recruits CD3 with a higher affinity than a human constant region [23] [33] . The fourth TCR modification is codon optimized. This TCR does not promote proper pairing, rather it increases the rate of protein synthesis which increases the chance of each alpha or beta chain pairing with its correct, respective chain [25] . The fifth modification is the leucine zipper TCR. By attaching heterodimerization motifs to the C-terminus on the alpha and beta chain, a leucine zipper is created. This high affinity coiled coil interaction increases and promotes the correct pairing between alpha and beta TCR chains [26]. The sixth and final TCR modification is the single chain TCR. Linkage of the $V\alpha$ - $V\beta$ - $C\beta$ regions creates a three-domain chain that can be coexpressed along with a single $C\alpha$ domain [30] [29]. Since neither of these unique chains can pair with an endogenous chain, this TCR modification promotes proper pairing. A graphic representation of these different TCR modifications is shown in Figure 5.

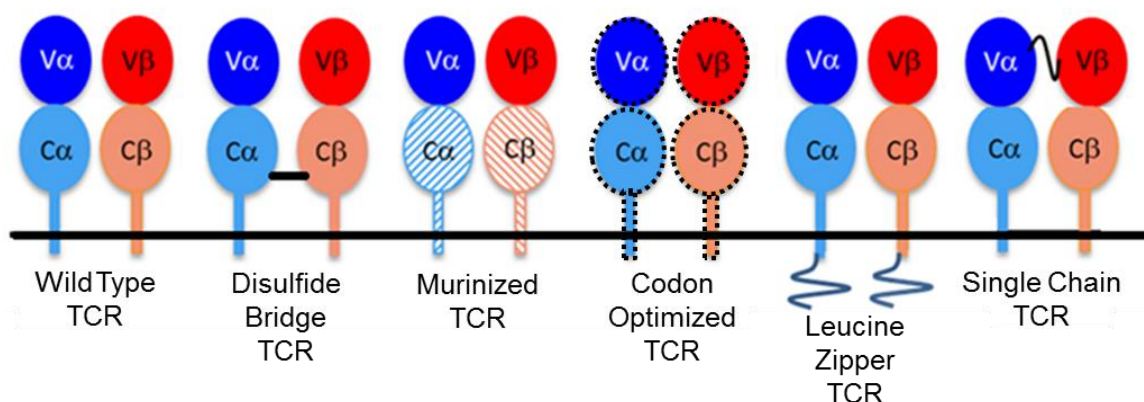


Image modified from Wang and Nishimura (2013) [3].

Figure 5. Structure of modified TCR constructs designed to prevent mispairing.

Shown above are the TCR modifications that were constructed and analyzed in this study. Disulfide bond linked TCR introduces another disulfide bond in the constant regions. The murinized TCR replaces human constant regions with mouse constant regions. Codon optimization of the TCR allows for an increase in protein synthesis. Leucine zipper TCR includes two heterodimerization motifs linked to C-terminus of the alpha and beta chains. Lastly, the single chain TCR links the two variable regions with the beta constant region, leaving the constant alpha region as a single domain. All of these modifications were constructed under the hypothesis that they will increase surface expression and function in comparison to wild type and therefore make a better T cell.

Constructing Retroviral Vector Encoding Modified T Cell Receptors

Our lab uses a modified SAMEN retroviral construct to introduce TCR genes into T cells. The structure of this vector is shown in Figure 6. At the 5' end of the vector is a human cytomegalovirus (CMV) long terminal repeat (LTR) promoter. This promoter allows for enhanced constitutive levels of transcription. Following this promoter is a splice donor and splice acceptor site for RNA splicing. Ψ is the packing signal used to direct viral DNA into virions [34]. The TCR alpha gene and TCR beta gene are linked by a P2A self cleaving peptide. This allows the alpha and beta chains to be synthesized in a 1:1 ratio. Following the TCR beta gene is a T2A self cleaving peptide followed by CD34t which is synthesized in a 1:1 ratio with the TCR chains. CD34t is a truncated CD34 molecule and consequently lacks its intracellular signaling domain [35]. This is a unique marker of transduction and is beneficial for a number of reasons. There is nothing that limits surface expression of CD34t, therefore its expression levels are analogous to the amount of TCR protein being made. CD34t can also be used to sort transduced cells which allows for an easy method of attaining a pure transduced T cell population. Following the CD34t, is a 3' LTR sequence for genomic insertion.

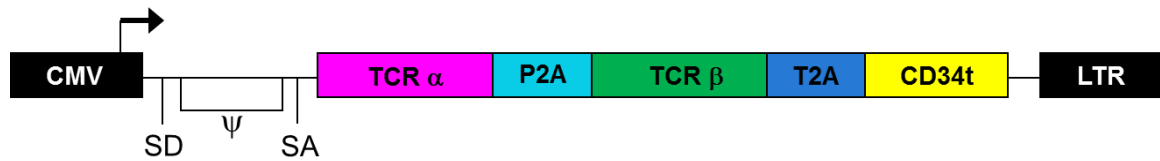


Figure 6. Structure of the retroviral vector used for TCR gene transfer.

A modified SAMEN retroviral vector was used to transfer TCR genes to alternate effectors. The retroviral vector used in this study contains a CMV promoter, splice donor (SD) and splice acceptor (SA), psi (ψ) packaging signal, HCV 1406 TCR alpha (α) and beta (β) chains, CD34t, and long terminal repeats (LTR). The HCV 1406 TCR α and β genes and the CD34t molecule are linked via P2A and T2A sequences, respectively. P2A and T2A are self-cleaving peptides resulting in three separate proteins. CD34t is used as a marker for transduction.

Aside from the disulfide bridge TCR construct, all other modified TCRs were chemically synthesized and provided in a pUC57 plasmid by GenScript. A two step process was used to insert the HCV 1406 TCR/CD34t cassette in the SAMEN vector. First, the TCR/CD34t cassette was built in pCR2.1. Then the TCR/CD34t cassette was subcloned into SAMEN. In order to be sure subcloning and ligations into the pCR2.1 and SAMEN vectors were correct, restriction enzyme digestion analysis for correct band length patterns was done prior to gel purification of correct band lengths. NotI, EcoRI, and BamHI digested pCR2.1 DNA should reveal three bands corresponding to the TCR (1.8 kb), CD34t (1.0 kb), and the pCR2.1 backbone (4.0 kb). An example of this gel is shown in Figure 7. Correct band lengths are shown in lanes 1 and 7 representing the correct recombinant clones. The same process was done with recombinant clones transformed into *E. coli* with the plasmid containing the SAMEN vector ligation product. As predicted, the correct clones reveal five bands corresponding to the TCR (1.8 kb), CD34t (1.0 kb), and fragments of the SAMEN backbone (0.3 kb, 1.8 kb, and 3.0 kb). An example of this gel is shown in Figure 8. Correct band lengths are shown in lanes 1,3,4,6, and 7 representing the correct recombinant clones. Final retroviral constructs products were also sequenced to assure no DNA base errors occurred.

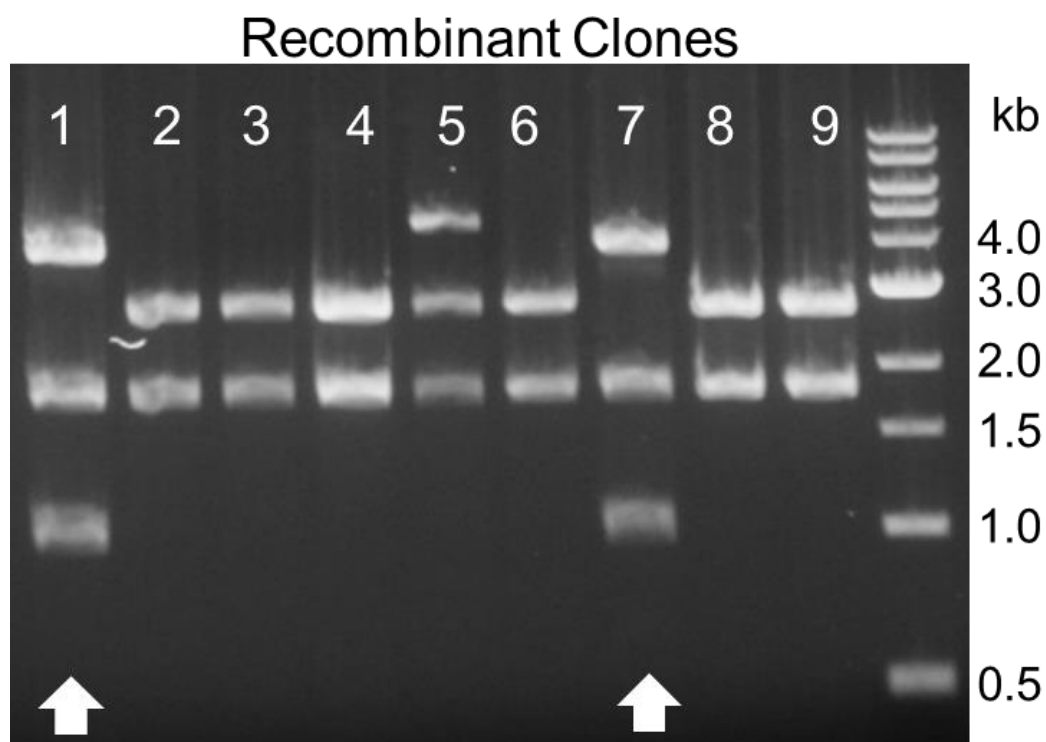


Figure 7. Restriction enzyme digestion analysis of recombinant pCR2.1 clones.

DNA from nine recombinant clones, potentially containing the TCR in pCR2.1, was digested with NotI, EcoRI, and BamHI enzymes. The correct band lengths should be 1.0 kb, 1.8 kb, and 4.0 kb. This is consistent with the size of these DNA fragments that are referenced in Figure 3. Correct recombinant clones are indicated by the white arrows.

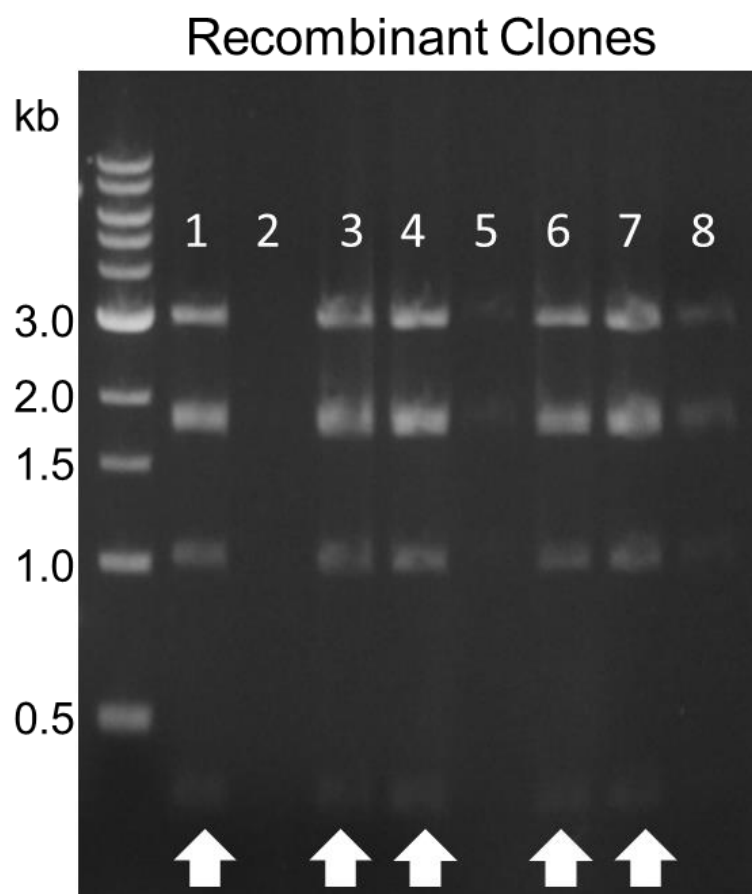


Figure 8. Restriction enzyme digestion analysis of recombinant SAMEN clones.

DNA from eight recombinant clones, potentially containing the TCR/CD34t in SAMEN, was digested with NotI, EcoRI, and BamHI enzymes. The correct band lengths should be 0.3 kb, 1.0 kb, 1.8 kb (2 bands) and 3.0 kb. This is consistent with the size of these DNA fragments that are referenced in Figure 3. Correct recombinant clones are indicated by the white arrows.

Generating PG13 Producer Cell Lines

A two step process was used to make PG13 producer cell lines. HEK293GP supernatant was generated and used to retrovirally transduce PG13 cells to create a high titer retroviral producer cell line. Eight different PG13 cell lines were made, each producing a different retrovirus containing either the wild type HCV 1406 TCR, the six different HCV 1406 modified TCRs, or a TIL 1383I melanoma TCR that was used as a negative control in functional assays. The resulting PG13 cell lines were sorted on a FACSAria by their CD34 expression to achieve a pure population of CD34⁺ PG13 cells. Histograms of the eight PG13 producer cell lines post CD34 sort is shown in Figure 9. As evident in the figure, each PG13 cell line represents a pure and uniform CD34⁺ population ranging from 97.3% to 100% CD34⁺. These pure CD34⁺ cell populations were then used to make high titer retroviral supernatant for T cell transduction.

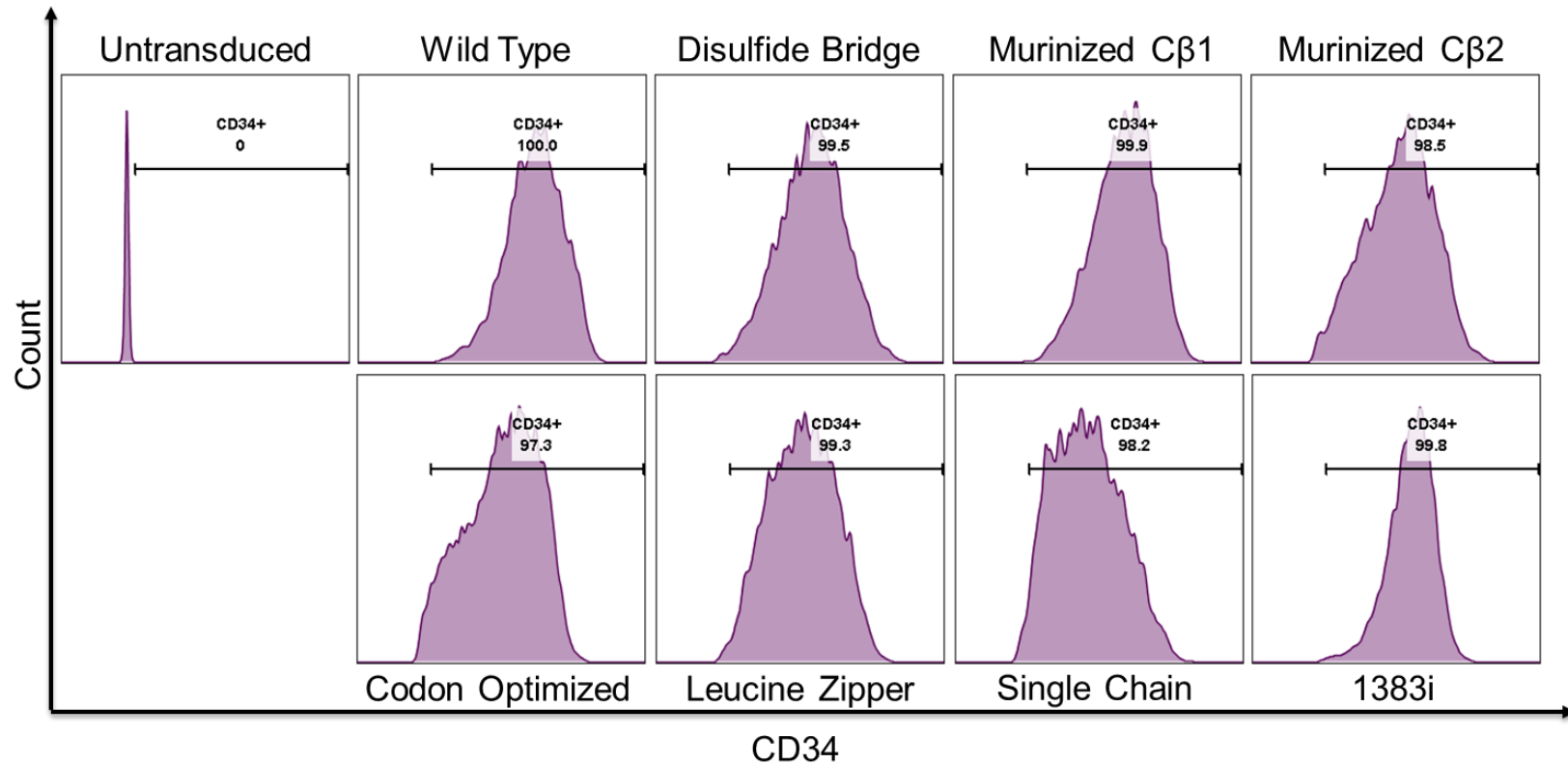


Figure 9. CD34 surface expression on PG13 producer cells post CD34 purification.

PG13 cell lines were sorted by CD34 to achieve a pure CD34⁺ population. Untransduced and transduced PG13s were labeled with an anti-CD34 mAb. Each histogram represents the relative log fluorescence of 1×10^6 cells as measured by flow cytometry.

PBMC Transductions

PBMCs from three healthy donors were transduced with each of the modified HCV 1406 TCRs and cultured with IL-2 and IL-15 to promote T cell growth. Three donors were used to control for donor variability. These transductions resulted in each of the three donors having a population of untransduced T cells, T cells transduced with the wild type HCV 1406 TCR, T cells transduced with each of the modified HCV 1406 TCRs, and T cells transduced with the TIL 1383i TCR. On day 7, these transduced T cells were fluorescently labeled with an anti-CD34-PE mAb and analyzed by flow cytometry to verify an efficient transduction of the retrovirus. Transduction efficiency among the 3 donors varied from 16% to 67% as measured by CD34 expression (data not shown). A representative example of transduction efficiency is shown in histograms in Figure 10 for donor 1 (donor 2 and donor 3 data not shown). As measured by CD34, transductions ranged from 25% to 66% in this example. T cells were then column sorted using CD34 magnetic beads to achieve a more pure and uniform transduced T cell population. Post CD34 bead sort, the CD34 purity among the 3 donors ranged from 72% to 93% (data not shown). Histograms representing each of donor 1's retrovirally transduced cell populations post bead sort are shown in Figure 11 as a representative example (donor 2 and donor 3 data not shown). As apparent in Figure 11, these different T cell populations now exhibit a more pure and uniform CD34⁺ population than in Figure 10. This CD34⁺ T cell population now ranges from 73% to 83%.

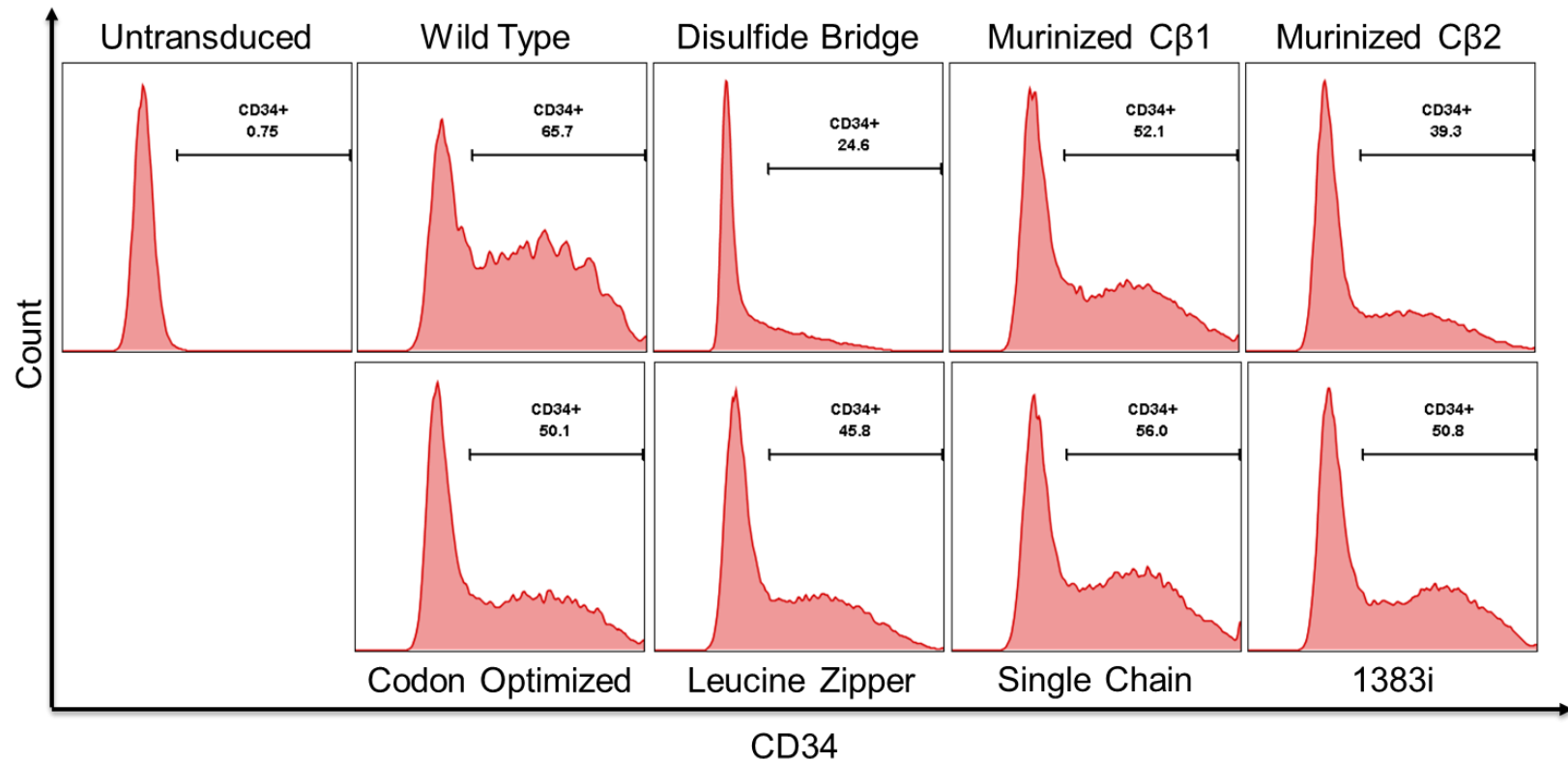


Figure 10. Donor 1 retrovirally transduced T cell populations.

On day 7, untransduced and transduced T cells were labeled with an anti-CD34 mAb to assure each population was efficiently retrovirally transduced. Each histogram represents the relative log fluorescence of 1×10^6 cells as measured by flow cytometry.

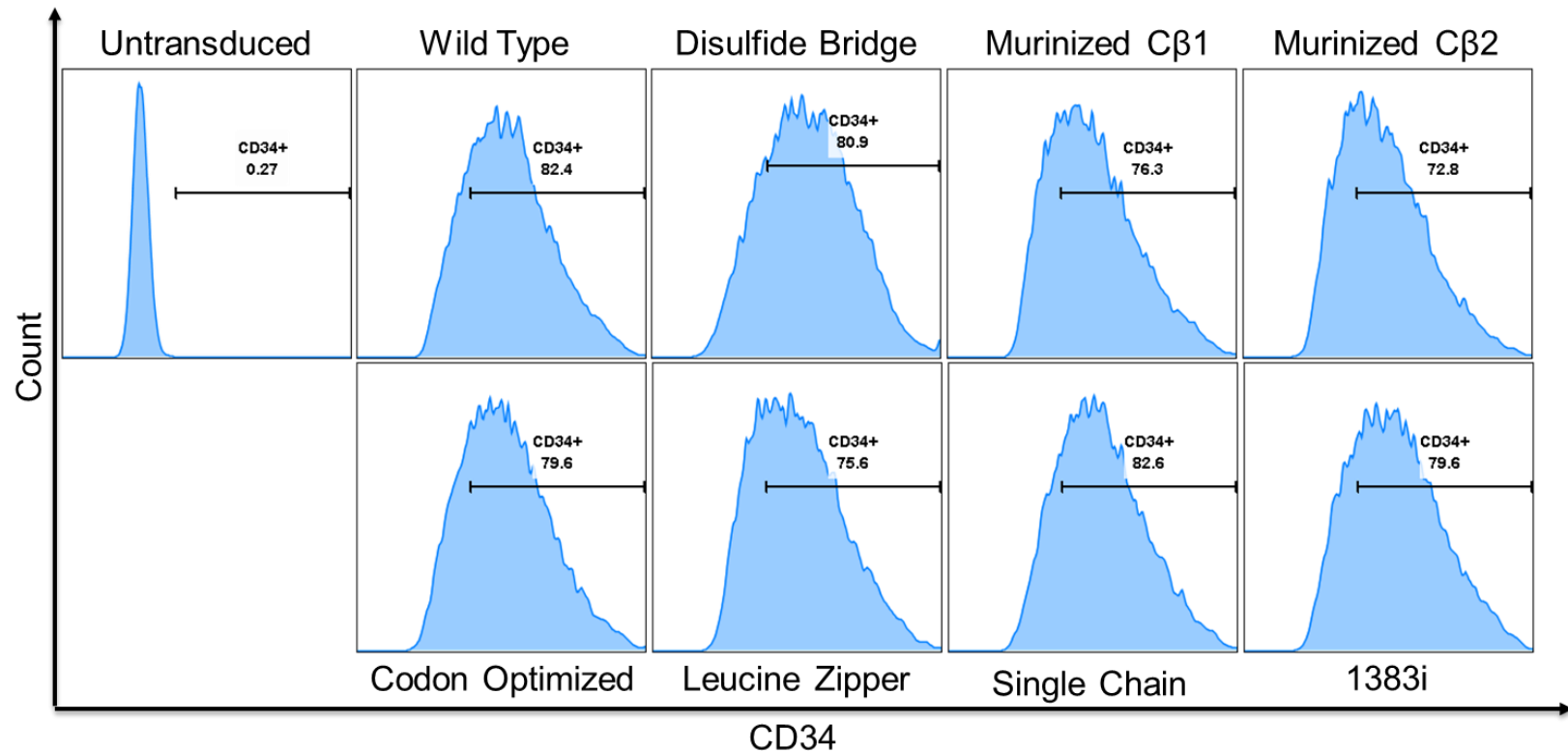


Figure 11. Donor 1 transduced T cell populations post CD34 magnetic bead sort.

On day 10, untransduced and transduced T cells were labeled with an anti-CD34 mAb to assure CD34 bead sort resulted in a more uniform and pure CD34 T cell population. Each histogram represents the relative log fluorescence of 1×10^6 cells as measured by flow cytometry.

As a strategy to promote proper TCR chain pairing, we hypothesized that these six TCR modifications will have a higher level of cell surface expression in comparison to the wild type TCR. Using the CD34 purified transduced T cells shown previously, we fluorescently labeled these cells with an anti-CD3 mAb, anti-CD34 mAb, and a HCV 1406 dextramer. This dextramer is unique in that it is a recombinant HLA-A*0201 molecule folded around the HCV NS3:1406-1415 peptide, therefore this dextramer will only bind properly paired introduced HCV 1406 TCRs. The flow cytometry plots are shown in Figure 12. Similar plots were generated for all 3 donors in 3 independent experiments (data not shown) but since all plots revealed similar results, one representative example is shown in Figure 12. All cells were gated on a CD3⁺ cell population in order to analyze only T cells. Negative gates are based on the fluorescence of untransduced T cells and TIL 1383I TCR transduced T cells were used to show the specificity of the HCV 1406 dextramer.

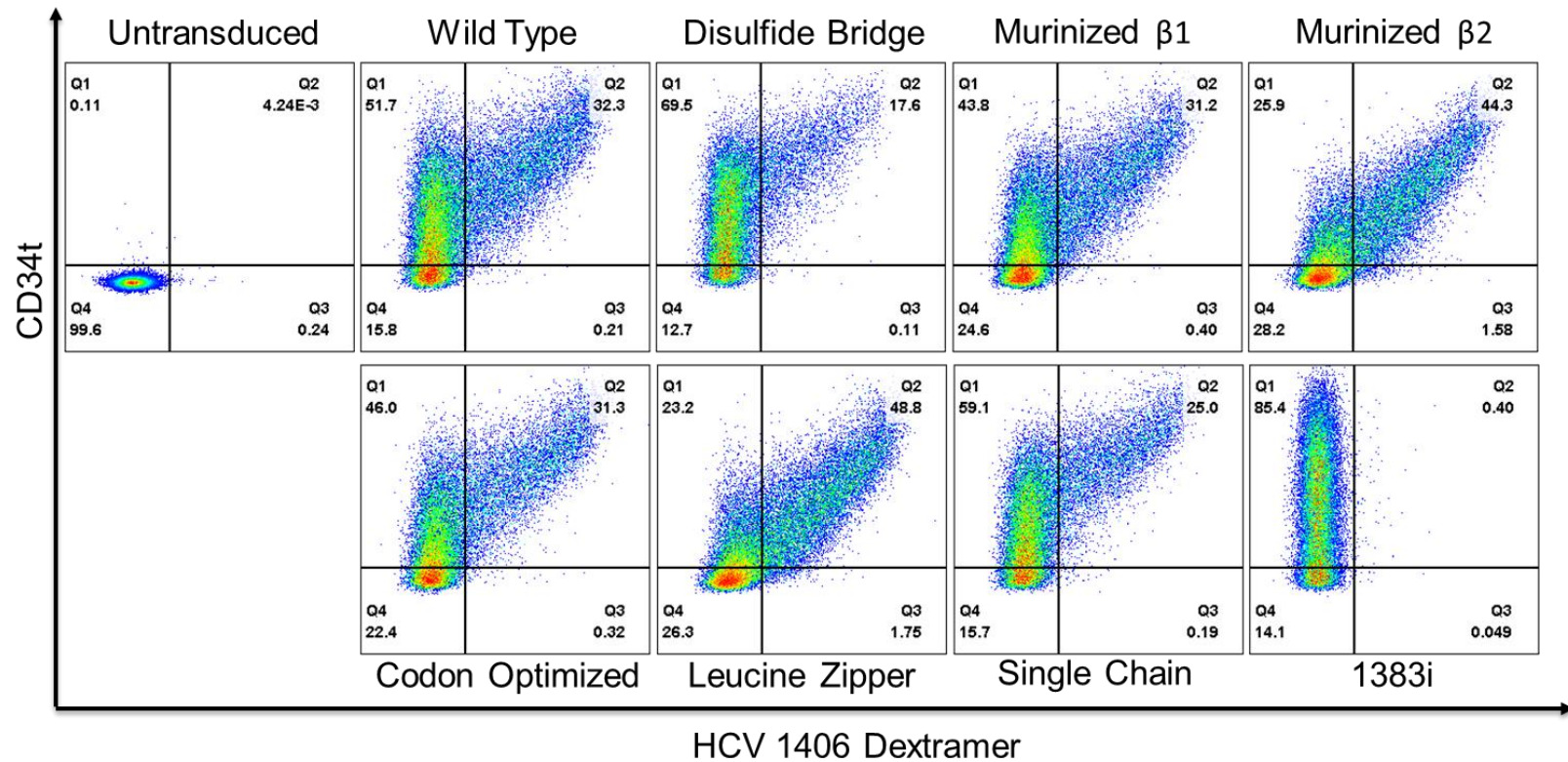


Figure 12. Cell surface phenotype of T cells transduced with T cells transduced with wild type HCV 1406 TCR or each modified HCV 1406 TCR.

Donor 1, experiment 1 untransduced and transduced T cells were immunofluorescently stained with anti-CD3 mAb, anti-CD34 mAb, and HCV 1406 dextramer. Each plot represents 5×10^4 collected events as measured by flow cytometry.

As shown in Figure 12, both Q1 and Q2 represent transduced T cells as measured by CD34 staining. Additionally, Q2 and Q3 represent T cells expressing the properly paired HCV 1406 TCR as measured by dextramer staining. Therefore, by dividing Q2 by Q1+Q2 we calculate the percentage of cells that express properly paired HCV 1406 TCRs among the transduced T cell population. Table 1 is a representative example of the results we saw in all 3 donors across all 3 experiments. Based on this data, we generally saw about 20%-68% of the transduced T cells were also dextramer positive meaning they expressed the properly paired HCV 1406 TCR.

TCR	Dextramer ⁺ of CD34 ⁺
Wild Type	38.5%
Disulfide Bridge	20.2%
Murinized C β 1	41.6%
Murinized C β 2	63.1%
Codon Optimized	40.5%
Leucine Zipper	67.8%
Single Chain	29.7%

Table 1. Percentages of transduced T cells that express properly paired HCV 1406 TCRs.

The table shown above quantifies the amount of properly paired HCV 1406 TCRs expressed on T cells transduced with each of the modified TCRs. Q1 and Q2, as shown in Figure 11, represent the population of transduced T cells and Q2 alone represents transduced T cells that are also dextramer positive (for donor 1, experiment 1). Therefore, by dividing Q2 by Q1+Q2 we calculate the percentage of dextramer positive cells among the transduced T cells population as a whole. These calculated percentages are shown above for each of the T cells transduced with the different TCR modifications for donor 1 experiment 1.

These data reveal that T cells transduced with the disulfide bridge TCR and the single chain TCR have a lower percentage of properly paired TCRs expressed on the cell surface in comparison to the wild type TCR. The T cells transduced with the murinized C β 1 TCR and codon optimized TCR have a similar percentage of properly paired TCRs expressed on the cell surface in comparison to T cells transduced with the wild type TCR while T cells transduced with the murinized C β 2 TCR and leucine zipper TCR have more properly paired HCV 1406 TCRs expressed on the cell surface. By looking at the data in this quantitative way, it appears that the murinized C β 2 TCR and leucine zipper TCR promote proper pairing due to the increased levels of TCR expression on transduced cells. Additionally, it appears that the murinized C β 1 TCR and codon optimized TCR do not promote proper pairing since they had negligible expression differences from the wild type TCR. Lastly, it appears that the disulfide bridge TCR and the single chain TCR do not promote proper TCR chain pairing and could possibly hinder it due to the lower than wild type TCR expression levels on transduced T cells. These trends in the expression of properly paired HCV 1406 TCRs among the T cells transduced with the different TCRs was shown to be reproducible among the 3 donors and 3 experiments (data not shown). Both the murinized C β 2 TCR and leucine zipper TCR agree with our hypothesis that these TCR modifications will promote proper TCR chain pairing and therefore have a higher level of cell surface expression in comparison to the wild type TCR.

In addition to comparing the percentages of properly paired HCV 1406 TCRs among transduced T cells, it is important to also analyze the relationship between CD34 expression and properly paired TCRs expressed on a per T cell basis. Therefore, the Q2 quadrant of these flow plots shown in Figure 12 is important to focus on because this population represents transduced T cells that express the properly paired introduced HCV 1406 TCR. As shown in Figure 12, the Q2 quadrants vary in appearance between each HCV 1406 TCR modification. It is evident that the disulfide bridge HCV 1406 TCR and the single chain HCV 1406 TCR need a higher level of CD34 expression to achieve HCV 1406 TCR expression in comparison to the wild type HCV 1406 TCR, which can be interpreted by the shift up the Y axis in the disulfide bridge HCV 1406 TCR and single chain HCV 1406 TCR cell populations in Q2. There is a shift down in the murinized C β 2 HCV 1406 TCR and leucine zipper HCV 1406 TCR cell populations indicating the need for less CD34 expression to achieve HCV 1406 TCR expression. Another way to compare all these double positive quadrants together is to plot fitted lines derived from the compensated log X,Y values collected for each HCV 1406 TCR. Figure 13 combines data from all 3 donors and all 3 experiments to create fitted lines for each transduced T cell line that express properly paired modified or wild type HCV 1406 TCRs. It is important to point out that the expression of properly paired HCV 1406 TCRs on the cell surface is reproducible among the 3 donors, as shown in Figure 13.

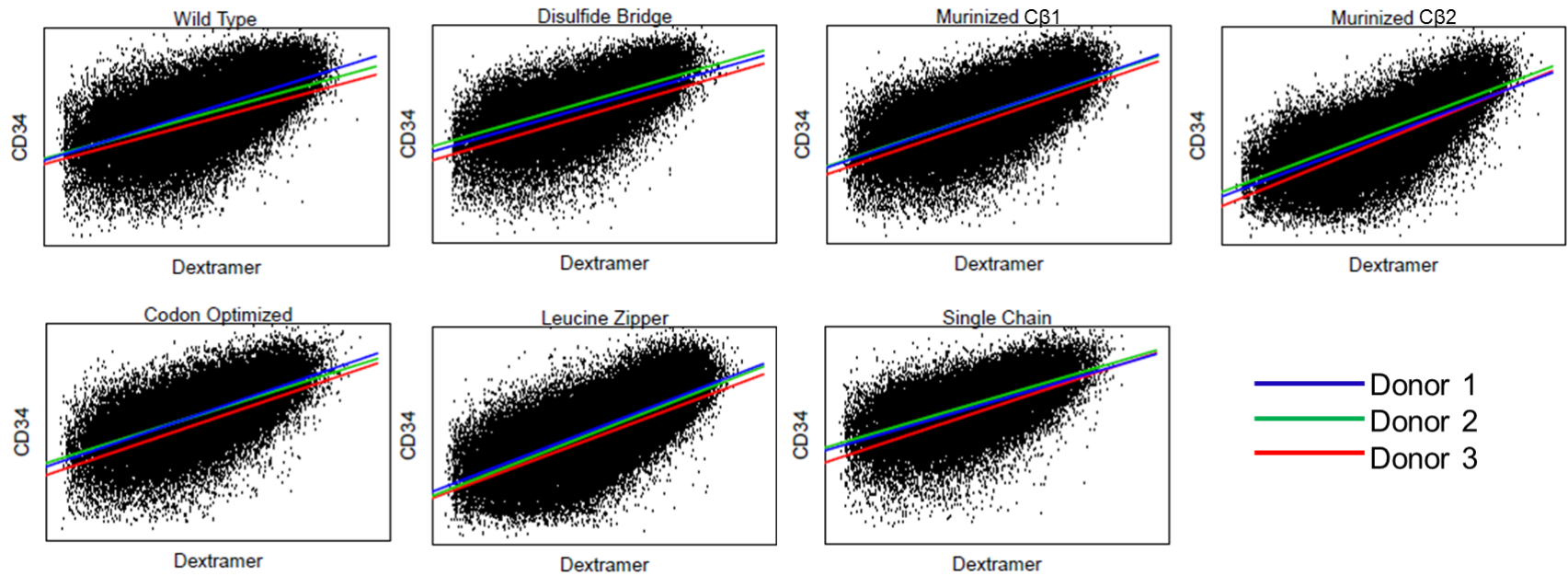


Figure 13. Comparison of donors among transduced reactive T cells transduced with wild type HCV 1406 TCR or modified HCV 1406 TCR.

The plot above shows the overlapping of fitted lines calculated from the double positive quadrant of each donor and experiment. Using the compensated log X,Y values from this quadrant for each transduced T cell line, donor, and experiment, fitted lines were plotted as shown above to show reproducibility among donors. Donor 1 is represented by the blue line, donor 2 the green line, and donor 3 the red line.

It is evident that the fitted lines for each donor have similar Y-intercepts and slopes for each TCR in Figure 13. This indicates that expression levels of properly paired HCV 1406 TCRs are consistent among different donors. Since donor reproducibility was evident, Figure 14 combines all the 3 donor and 3 experiments into one fitted line for each modified TCR. By looking at the Y-intercepts of the fitted lines for each HCV 1406 TCR, it is evident that properly paired murinized C β 2 HCV 1406 TCRs and properly paired leucine zipper HCV 1406 TCRs need much less transgene expression, as measured by CD34, than properly paired wild type HCV 1406 TCRs in order to express on the cell surface. This is shown by the lower levels of CD34 expression needed to achieve dextramer expression. Conversely, the single chain HCV 1406 TCR and the disulfide bridge HCV 1406 TCR appear to need higher levels of transgene expression to achieve the same levels of properly paired HCV 1406 TCRs on the cell surface, as measured by dextramer staining. Also, slightly lower transgene expression is needed for properly paired murinized C β 1 HCV 1406 TCRs or properly paired codon optimized HCV 1406 TCRs to express on the cell surface in comparison to the wild type HCV 1406 TCR. These trends continue as you follow the fitted lines of each HCV 1406 TCR. The lower transgene expression needed for properly paired murinized C β 2 HCV 1406 TCRs or properly paired leucine zipper TCRs could be a result of their respective gene modifications hypothesized to increase proper TCR chain pairing.

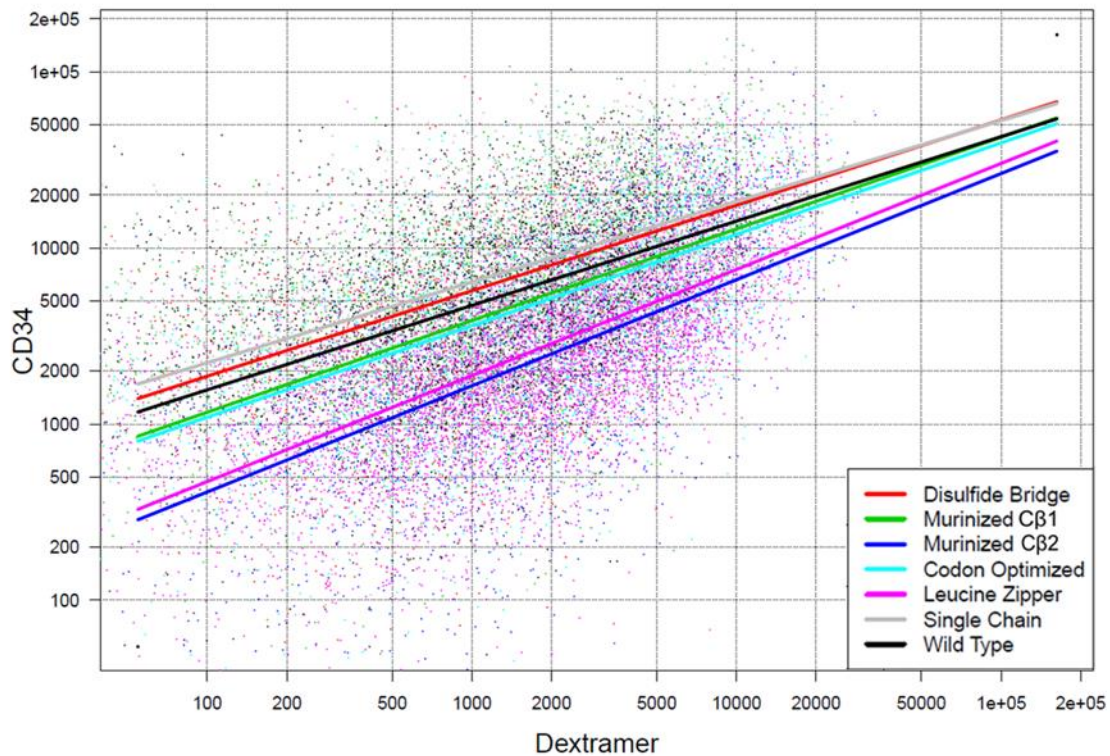


Figure 14. Comparison of transduced reactive T cells transduced with wild type HCV 1406 TCR or modified HCV 1406 TCR.

The plot above shows the overlapping of fitted lines calculated from the double positive quadrant of each donor and experiment. Using the compensated log X,Y values from this quadrant for each transduced T cell line, donor, and experiment, fitted lines were plotted as shown above.

Modified T Cell Receptor Function

We hypothesized that the modified TCRs used in this study will help prevent TCR chain mispairing between the introduced TCR and the endogenous TCR leading to an increase in properly paired TCRs and an increase in TCR expression. Consequently, we believe this increase in properly paired TCRs and increase TCR expression will result in an increase in T cell function. As shown above, T cells transduced with the murinized C β 2 TCR or the leucine zipper TCR appeared to have an increase in HCV 1406 TCR cell surface expression. T cells transduced with the disulfide bridge TCR or the single chain TCR appeared to have a decrease in HCV 1406 TCR cell surface expression, and T cells transduced with the murinized C β 1 TCR or the codon optimized TCR appeared to have equivalent HCV 1406 TCR cell surface expression when compared to the wild type TCR. Therefore, we would predict that T cells transduced with the murinized C β 2 TCR or the leucine zipper TCR will show an increase in T cell function, T cells transduced with the murinized C β 1 TCR, codon optimized TCR, or wild type TCR will function similarly, and lastly, T cells transduced with the disulfide bridge TCR or single chain TCR will show a decrease in T cell function. To determine if these HCC 1406 TCR cell surface expression levels correlate to T cell function and to determine if there is a TCR modification that functions significantly better than others, cytokine release assays were done to measure the amount of IFN- γ released upon antigen stimulation. Each transduced T cell line was cocultured with T2 cells loaded with the HCV 1406 peptide, irrelevant Tyrosinase peptide, HepG2:NS3⁻ tumor cells or HepG2:NS3⁺ tumor cells, which

is the HepG2 tumor line engineered to express the full length HCV NS3 protein.

These cocultures were performed in 3 independent experiments with each of the HCV 1406 TCR transduced T cells from 3 normal donors. IFN- γ production was measured as a marker for T cell activation because it is secreted by both CD4⁺ and CD8⁺ T cells and expression of IFN- γ producing genes occurs shortly after activation. The reactivity of T cells transduced with each modified TCR stimulated with peptide loaded T2 cells are shown in Figure 15. Due to the large number of samples with 3 donors and 3 experiments, Figure 15 shows a representative example of the peptide MHC reactivity (all other experiments are shown in Appendix A). This data shows that all the modified TCRs are very reactive against the specific HCV NS3:1406-1415 peptide in the context of MHC, and not the irrelevant Tyrosinase peptide. Generally, T cells transduced with the different TCR modifications resulted in T cells that functioned similar or worse than T cells transduced with the HCV 1406 wild type TCR. Occasionally T cells transduced with a modified TCR resulted in an increase in IFN- γ release in comparison to T cells transduced with the wild type HCV 1406 TCR. Since these cocultures use T2 cells saturated with the HCV 1406 peptide as stimulators, the amount of antigen present in the coculture could overcompensate for any reactivity or functional hindrances of the TCRs. Consequently, it is difficult to make overarching conclusions about comparisons between the different modified TCRs and the wild type TCR because there is so much variability between donors and experiments.

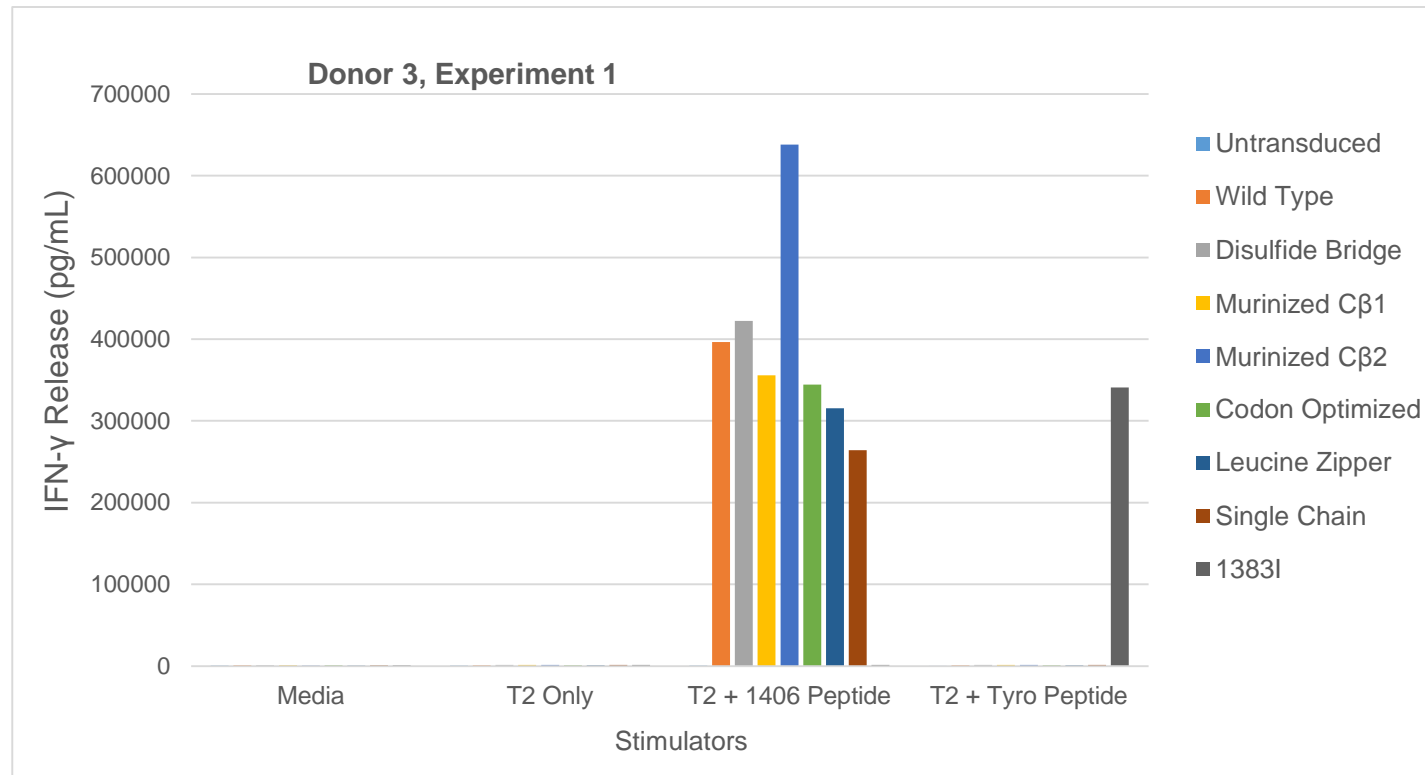


Figure 15. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 μ g/mL of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN- γ release was measured by ELISA.

In addition to peptide loaded targets, antigen reactivity stimulated by tumor cells was measured. The reactivity of HCV 1406 transduced T cells that were cocultured with HepG2:NS3⁺ and HepG2:NS3⁻ tumor cells is shown in Figure 16. Due to the large number of samples with 3 donors and 3 experiments, Figure 16 shows a representative example of tumor reactivity (all other experiments are shown in Appendix A). This data revealed that all the modified TCRs are reactive against the HepG2:NS3⁺ tumor cells. Generally, all T cells transduced with a modified HCV 1406 TCR functioned the same or better than T cells transduced with the wild type HCV 1406 TCR. The amount of IFN- γ released in the tumor cocultures appears to be significantly lower than in the cocultures with T2 cells pulsed with peptide. There is less naturally processed antigen on the surface of the tumor cells than the T2 cells that are exogenously loaded with antigen. This is because, a majority, if not all of the MHC complexes on T2 cells will present the exogenously loaded peptide. In contrast, a very small percentage of MHC on the surface of HepG2:NS3 will express our specific HCV 1406-1415 epitope. Despite the decrease in IFN- γ release, these tumor findings are more significant than the peptide MHC data because this tumor line will present naturally processed antigen and as a result is more biologically relevant in terms of what is actually occurring in an HCV infected cell.

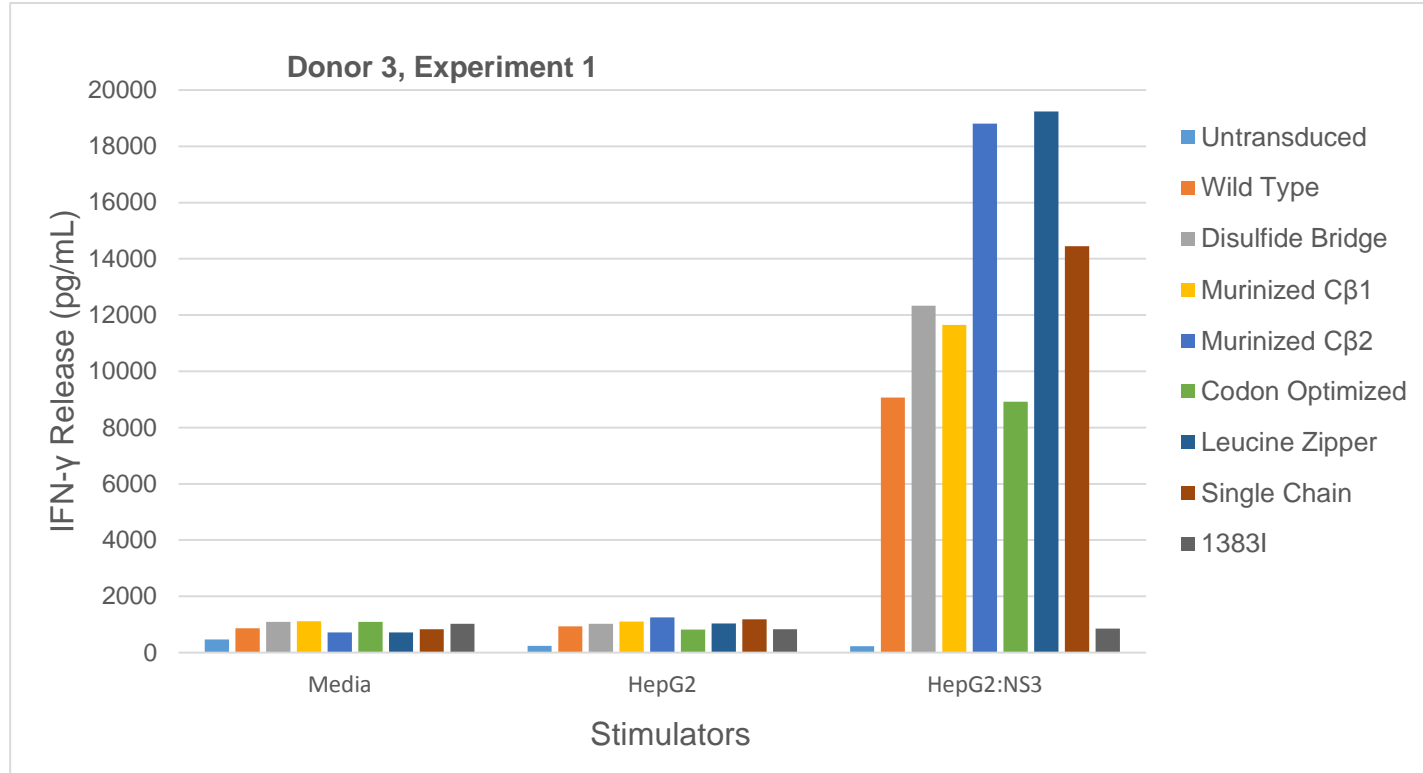


Figure 16. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN- γ release was measured by ELISA.

It is important to address the fact that the T cells put into these cocultures varied by CD34 purity as well as TCR cell surface expression depending on which TCR modification the cell was transduced with, as shown in Figure 11. This means that the number of actual reactive T cells in the cocultures is different for each transduced T cell line depending on donor, experiment, and TCR modification. Flow cytometry was done the same day that each of the cocultures were set up. Previously shown in Table 1 are the percentages of the T cells that express the properly paired TCR out of the CD34⁺ population as a whole for donor 1 experiment 1. Because this percentage represents the only T cell population that could possibly be reactive in this coculture system, we thought the IFN- γ values would be more telling if they were divided by these percentages. As a result, the value would be normalized to the percentage of transduced T cells that express the properly paired introduced TCR. For example, in donor 1 experiment 1 the percentage of CD34⁺ cells that also stained for dextramer is 38.5% for T cells transduced with the wild type TCR (Table 1). The IFN- γ value for donor 1 experiment 1 is 940 pg/mL for HepG2:NS3 reactivity. To calculate this normalized value, 940 was divided by 0.385 resulting in a value of 2,442 pg/mL. This new value is now a representation of the IFN- γ release specifically in terms of only the reactive T cells. This calculation was done for each condition, donor, and experiment. A representative side by side comparison of not normalized and normalized HepG2:NS3⁺ tumor data is shown in Figure 17.

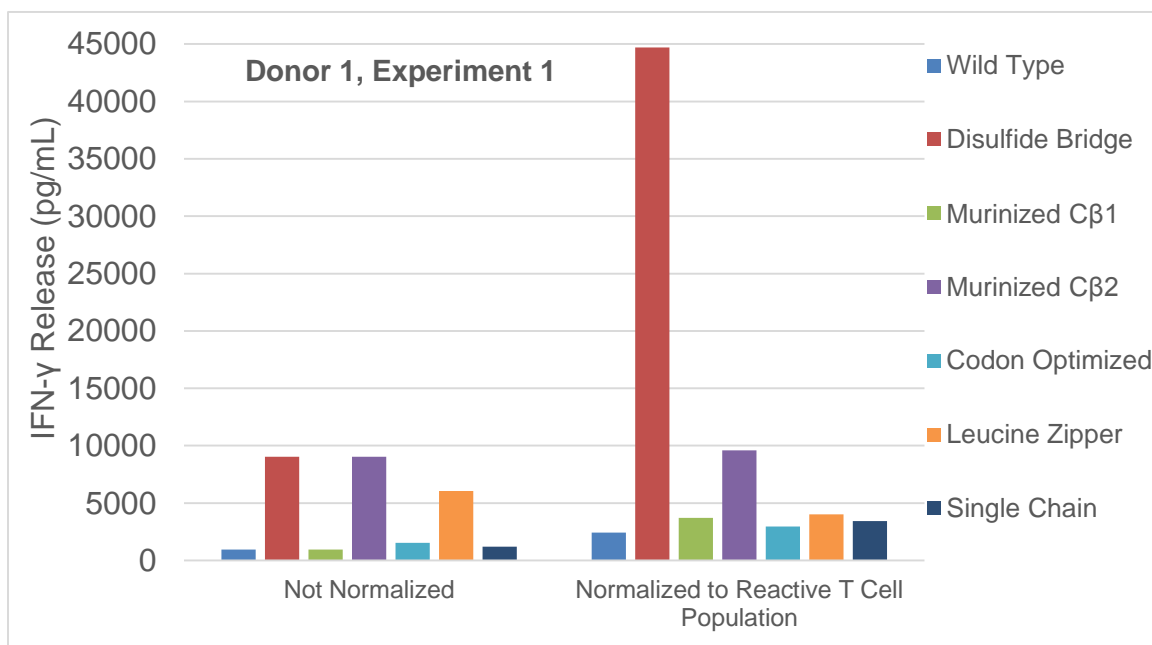


Figure 17. Comparison of HepG2:NS3 T cell reactivity normalized to percent of tumor reactive T cells.

The IFN- γ values for each condition and transduced T cell type in donor 1, experiment 1, were divided by the percentage of CD34 positive cells that were dextramer positive. This percentage of cells (shown in Table 1) represents the population of transduced T cells that are also reactive. The raw IFN- γ values that were not normalized are shown on the left in the graph above while the normalized values are shown on the right.

There are evident changes shown in Figure 17 when the data is normalized to represent the cytokine production from only reactive T cells. There is a large increase in the amount of IFN- γ released by the disulfide bridge TCR. This is because only 20.2% of transduced T cells are responsible for this cytokine production. The converse is shown for example in the leucine zipper TCR. This is because even with the large amount of cytokine being released, 67.8% of transduced T cells are reactive which is the highest percentage of all the TCR modified transduced T cells. As shown in Figure 17, by normalizing to the percentage of transduced T cells that can be reactive, the IFN- γ values can be representative of their corresponding levels of properly paired TCR expression. In conclusion, we believe that normalizing these IFN- γ values to the percentage of transduced T cells that are reactive is a better representation of the functional capabilities of these different transduced T cells.

In order to determine which TCR modifications have higher levels of cell surface expression or an increase in T cell function, we have been comparing each modification to the wild type TCR. Therefore, to be able to better visualize these functional differences (shown in Figures 15-16) between each modified TCR and the wild type TCR, it is beneficial to look at log fold changes when standardized to the reactivity of T cells transduced with the wild type HCV 1406 TCR. Log fold changes in IFN- γ release between the T cells transduced with the wild type TCR and the modified TCRs for the peptide and tumor data are shown in Figures 18-19, respectively.

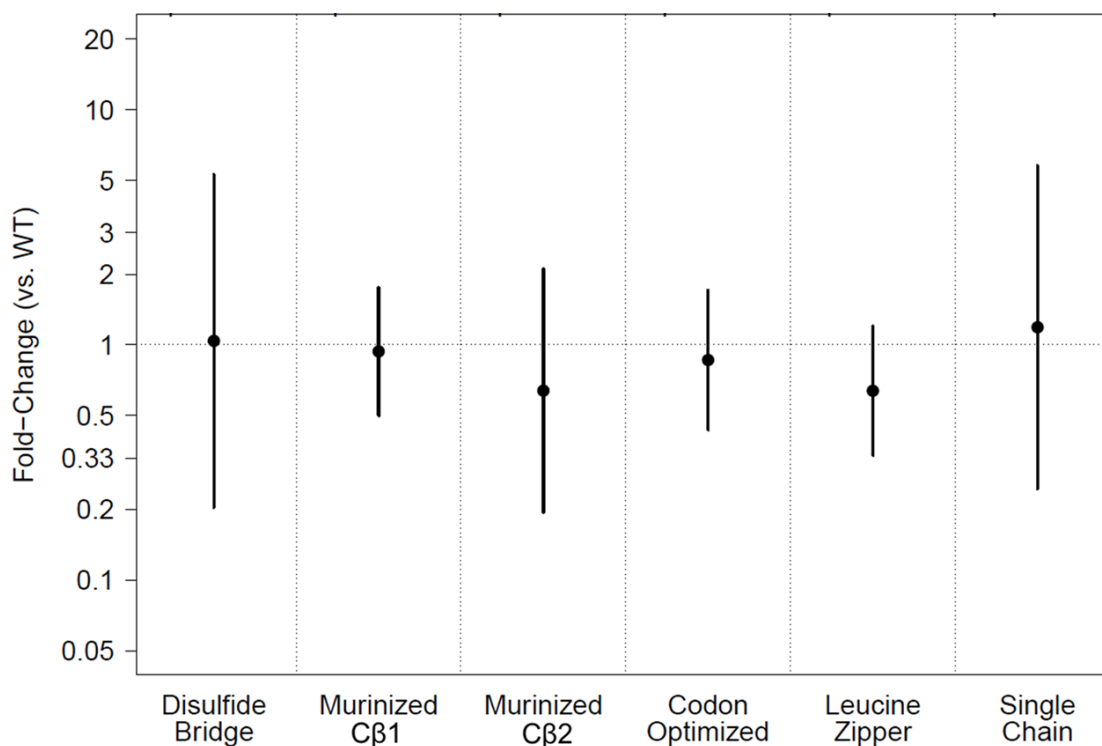


Figure 18. Log fold changes in peptide reactivity between T cells transduced with wild type HCV 1406 TCR and T cells transduced with modified HCV 1406 TCRs.

The graph shown above represents log fold changes of HCV 1406 peptide reactivity calculated between T cells transduced with a modified HCV 1406 TCR and T cells transduced with the wild type HCV 1406 TCR. This graph represents the data for all 3 donors in all 3 experiments. Linear combinations of model coefficients were calculated with 95% confidence intervals. Black lines represent the fold changes that fall within the 95% confidence interval among all donors and experiments. Black circles represent the median fold change among donors and experiments.

Figure 18 represents a unique way to compare HCV 1406 peptide reactivity between T cells transduced with each of the modified HCV 1406 TCRs and T cells transduced with the wild type HCV 1406 TCR across the 3 donors and 3 experiments. A linear regression was used to model the IFN- γ values by TCR, donor, and experiment. Linear combinations of model coefficients were calculated with 95% confidence intervals. Based on the fold changes shown in Figure 18, it is evident that none of the T cells transduced with a modified TCR have a significant difference in fold changes of peptide reactivity in comparison to the T cells transduced with the wild type TCR. This can be inferred by the black circles, which represent the median fold changes among donors and experiments. The black circles generally reside around 1 except for T cells transduced with the murinized C β 2 TCR or the leucine zipper TCR, which appear to be lower than 1. The black lines represent the ranges that the fold changes among donors and experiments for each TCR fall within a 95% confidence interval. T cells transduced with the disulfide bridge TCR, the murinized C β 2 TCR, or the single chain TCR appear to have a large variability as seen in the larger length of their respective black line. In conclusion, there is a decrease in peptide reactivity based on the fold changes seen in T cells transduced with either the murinized C β 2 TCR or the leucine zipper TCR when compared against T cells transduced with the wild type TCR. All other T cells transduced with the modified TCRs show no apparent differences in fold changes when compared to T cells transduced with the wild type TCR.

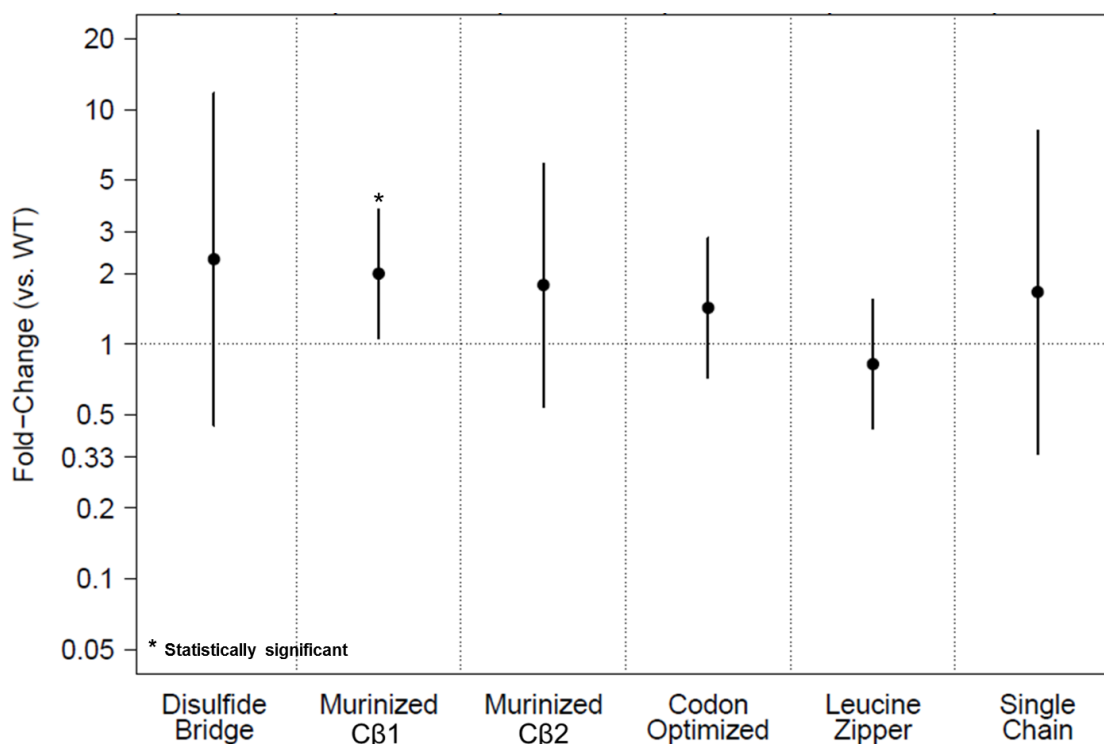


Figure 19. Log fold changes in tumor reactivity between T cells transduced with wild type HCV 1406 TCR and T cells transduced with modified HCV 1406 TCRs.

The graph shown above represents log fold changes of HepG2:NS3⁺ tumor reactivity calculated between T cells transduced with a modified HCV 1406 TCR and T cells transduced with the wild type HCV 1406 TCR. This graph represents the data for all 3 donors in all 3 experiments. Linear combinations of model coefficients were calculated with 95% confidence intervals. Black lines represent the fold changes that fall within the 95% confidence interval among all donors and experiments. Black circles represent the median fold change among donors and experiments.

In addition to comparing peptide reactivity, the same analysis was done to compare tumor reactivity between T cells transduced with the modified HCV 1406 TCRs and T cells transduced the wild type HCV 1406 TCRs among 3 donors and 3 experiments. Figure 18 represents the fold changes in IFN- γ reactivity against HepG2:NS3⁺ tumor cells. Except in T cells transduced with the leucine zipper TCR, it is evident that the median fold change in reactivity among all T cells transduced with a modified TCR is increased when compared to T cells transduced with the wild type TCR. This is different than what was seen in the peptide reactivity shown in Figure 18, where only T cells transduced with the single chain TCR appeared to have a slight increase in fold change above 1. We believe that Figure 19 is a better representation of actual T cell function because this coculture system used HepG2:NS3⁺ tumor cells as the stimulator. These tumor cells will present naturally processed antigen in contrast to peptide loaded T2 cells. Therefore, the HepG2:NS3⁺ tumor cells mimic an actual HCV infected hepatocyte. The increase in fold change seen in T cells transduced with the murinized C β 1 TCR is statistically significant since its whole range of fold changes falls above 1. Similar to data shown in Figure 18, the black lines represented by T cells transduced with the disulfide bridge TCR, the murinized C β 2 TCR, and the single chain TCR reveal a higher level of variability than T cells transduced with the murinized C β 1 TCR, the codon optimized TCR, or the leucine zipper TCR. Although generally not statistically significant, an increase in tumor reactivity represented by an increase in fold changes from T cells

transduced with the wild type TCR, is evident in T cells transduced with a modified TCR. One exception to this is the decrease in fold change shown in T cells transduced with the leucine zipper TCR.

CHAPTER FOUR

DISCUSSION

Modified T Cell Receptors to Enhance Proper Chain Pairing

One of the challenges in introducing another TCR into a T cell is the potential for alpha and beta chain mispairing between endogenous and introduced TCR chains. One approach in promoting proper TCR chain pairing in engineering modifications in the introduced TCR that will enhance proper pairing. This thesis focused on comparing six modifications that have been shown to promote TCR chain pairing. These modifications include introducing a disulfide bridge in the alpha/beta constant regions, substituting human with murine constant regions, codon optimization to enhance protein synthesis, TCR chain leucine zipper fusions, and a single chain TCR. Our retroviral vector used to deliver these TCR genes, expresses a unique CD34t transduction marker that is translated in a 1:1 stoichiometric ratio of TCR protein and CD34t. Therefore, by using this internal reference standard we have a unique way comparing these modified TCRs. We hypothesized that when compared to the wild type TCR, the modified TCRs will have higher levels of TCR cell surface expression resulting in a higher level of T cell function. These studies can also compare the different TCR modifications to determine if there is an optimal modification that would be beneficial in TCR gene modified T cells for adoptive T cell transfer.

Modified T Cell Receptor Cell Surface Expression

As a strategy to improve proper pairing of the introduced HCV 1406 TCR on the cell surface, we made six modified HCV 1406 TCRs. To determine if these modifications increased the levels of TCR cell surface expression in comparison to the wild type TCR, the transduced T cells were fluorescently labeled with anti-CD34 mAb to measure transduction efficiency and with an HLA-A*0201 dextramer folded around HCV NS3:1406-1415 peptide to measure properly paired HCV 1406 TCRs.

As shown in Figure 12 and quantified in Table 1, the murinized C β 2 TCR and leucine zipper TCR promote proper pairing due to the increased levels of HCV 1406 TCR expression on transduced cells. Additionally, it appears that the murinized C β 1 TCR and codon optimized TCR do not have an effect on pairing since they had negligible expression differences from the wild type TCR. Lastly, it appears that the disulfide bridge TCR and the single chain TCR do not promote proper TCR chain pairing and could possibly hinder it due to the lower than wild type TCR expression levels on transduced T cells.

The leucine zipper TCR has the highest level of properly paired TCRs on transduced T cells. It is also evident in Figure 13 that low levels of CD34 transgene expression are needed for higher levels of properly paired TCRs on the cell surface. These results could be a possible consequence of the leucine zipper formation that occurs when these alpha and beta chains pair together. The heterodimerization motifs of the c-Jun and v-Fos proteins present on the C

terminal ends of the alpha and beta chains, respectively, have a high affinity for each other and will result in a favorable heterodimer formation [27] [26]. It makes sense that lower levels of transgene expression are needed for higher levels of level dextramer staining because roughly 68% of all transduced T cells will express this properly paired TCR. In conclusion the addition of the leucine zipper on the alpha and beta TCR chains is an effective way to result increase TCR expression due to the increase in proper pairing.

In addition to the leucine zipper TCR, the murinized C β 2 TCR exhibits an increase in TCR cell surface expression in comparison to wild type. Generally, 63% of transduced T cells also express the properly paired HCV 1406 TCR. By substituting human with murine constant regions, a murinized TCR chain cannot pair with a human TCR chain and this essentially can eliminate the chance of potential mispairing [33]. This can attribute to the increase in properly paired TCRs. Additionally, it has been shown that murine constant regions have a higher affinity and thus stability with the CD3 ζ chain [23]. Not only can this stabilize the TCR on the cell surface, but can also increase the chances of this murinized C β 2 TCR to bind to the CD3 complex in the ER. Since there are two beta mouse constant regions, modified TCRs were made with each murine beta constant region. Surprisingly, generally only 42% of the T cells transduced with the murinized C β 1 TCR expressed the properly paired HCV 1406 TCR. This is significantly less than the murinized C β 2 and more comparable to T cells transduced with the wild type TCR. There are five amino acids in the murine beta

constant region are shown to be important for an increase in TCR surface expression [24]. These beta chain murine amino acids are K-18, A-22, I-133, A-136 and H-139 [24]. While both our murine beta chains contain the K-18 and A-22 amino acids, only the C β 2 construct contains the I-33, A-136, and H-139 amino acids. The decreased levels of TCR cell surface expression seen in the murinized C β 1 TCR in comparison to the murinized C β 2 TCR, could be a result of lacking the latter 3 important amino acids. Based on these results, it is evident that substituting human constants regions with murine constant regions, specifically the C β 2 region, is an effective way to enhance proper pairing of the introduced TCR chains.

Similar to the murinized β 1 TCR, the codon optimized TCR was expressed in levels comparable to the wild type TCR. The theory for codon optimization is that protein translation will be increased and therefore more introduced TCR protein will allow for an increase in the likelihood of proper pairing due to more protein being made and thus present in the ER [25]. The expression levels of the codon optimized TCR did not follow this hypothesis. One explanation could be that despite the increase in protein being made, there is no driving force to actually promote proper pairing due to the lack of a TCR gene modification. Therefore, codon optimization of the introduced TCR is not an effective way to increase the TCR cell surface expression levels.

Unlike the leucine zipper, murinized, and codon optimized TCR, the disulfide bridge and single chain TCR displayed lower levels of TCR expression

than the wild type TCR, with only about 20% and 30% of transduced T cells expressing the properly paired HCV 1406 TCR, respectively. Additionally, it was shown these two modified TCRs needed higher levels of transgene expression to maintain expression levels of properly paired HCV 1406 TCRs that were comparable to the wild type HCV 1406 TCR. One explanation for the low disulfide bridge HCV 1406 TCR expression could be that the modification only changes one amino acid in each alpha and beta chain. Therefore, there is not a large driving force for proper pairing. Also, because there is this additional cysteine in the alpha and beta chains, this cysteine could improperly pair with the cysteine present in the transmembrane domain. This could lead to improper formation of the TCR if the constant region of one chain potentially forms a disulfide bond with the transmembrane domain of another chain via their cysteine residues. The single chain TCR links the two variable regions a 15-residue glycine linker to allow for correct folding. The three-domain TCR ($V\alpha$ - $V\beta$ - $C\beta$) is made as a separate protein from the $C\alpha$ single domain [29]. The proper folding of this three-domain TCR may not be favorable despite the linker. In conclusion, due low expression of properly paired disulfide bridge HCV 1406 TCRs and properly paired single chain HCV 1406 TCRs, these gene modifications are not an effective way to augment proper TCR chain pairing.

Modified T Cell Receptor Transduced T Cell Function

It has been reported that six different TCR gene modifications would enhance proper TCR chain pairing and thus increase levels of HCV 1406 TCR cell surface expression and consequently result in increased T cell function. Based on the levels of TCR cell surface expression, we would predict that T cells transduced with the leucine zipper TCR and the murinized C β 2 TCR would function the best, T cells transduced with the murinized C β 2 TCR and the codon optimized TCR would function similar to T cells transduced with the wild type TCR, and lastly, T cells transduced with the disulfide bridge TCR or the single chain TCR would function worse. It is evident from our data that this hypothesis does not always hold true. There are a couple of potential explanations as to why expression of properly paired HCV 1406 TCRs on the surface cell surface did not always correlate to T cell function.

T cells transduced with the leucine zipper HCV 1406 TCR expressed the highest levels of properly paired HCV 1406 TCRs and with low transgene expression. Despite this, when the IFN- γ release values were standardized to represent reactive T cells, the tumor data revealed that T cells transduced with this TCR were the only transduced T cells to have the median in fold changes in tumor reactivity lower than T cells transduced with the wild type HCV 1406 TCR. One explanation for this is that not all the properly paired leucine zipper TCRs on the surface were actually able to function. The leucine zipper construct involved the addition of 40 extra amino acids on the alpha and beta chains and

consequently there could be steric hindrance among these molecules, the CD3 ζ chain, and incoming signaling molecules. Since a TCR must associate with the CD3 complex before transport to the cell surface, it could be that the leucine zipper fusion proteins do not hinder binding to the CD3 complex due to high levels of properly paired HCV 1406 TCRs, but rather could be problematic for signaling molecules such as Lck and ZAP70 and subsequent phosphorylation of the CD3 ζ chain. Our data supports the conclusion that high levels of properly paired leucine zipper HCV 1406 TCRs expressed on the cell surface may not correlate to T cell function due to steric hindrances in the signaling complex.

Additionally, we saw that the murinized C β 1 TCR required slightly less transgene expression than the wild type HCV 1406 TCR to express properly paired TCRs on the cell surface and also a very slight increase in properly paired HCV 1406 TCRs on transduced T cells. These expression levels were still lower than the levels of the murinized C β 2 TCR, yet in some ways T cells transduced with the murinized C β 1 TCR seemed to function better when stimulated with tumor cells than T cells transduced with the murinized C β 2 TCR. For example, in Figure 19 the range of fold changes in T cells transduced with the murinized C β 1 TCR never falls below the 1, where the range for T cells transduced with the murinized C β 2 TCR does. Also, the range of variability shown in T cells transduced with the murinized C β 1 TCR is smaller than T cells transduced with the murinized C β 2 TCR, meaning there is more consistency between donors and experiments among the T cells transduced with the murinized C β 1 TCR. An idea

for high levels of properly paired murinized C β 2 TCRs not correlating to function, could be that there were not enough signaling molecules available to accommodate all the reactive TCRs. All the signaling molecules necessary for the cascade of TCR activation via signaling through CD3 all are involved in various feedback loops in order to keep a T cell from activating out of control. Since our HCV 1406 TCR is classified as a high affinity TCR, any TCR gene modifications to increase proper pairing, TCR expression, and T cell function will increase the responsiveness of the T cell in addition to already expressing a high affinity TCR. Therefore, having high levels of a high affinity TCR expressed on the cell surface might not subsequently result in increased T cell function. When T cells express the high affinity HCV 1406 TCR, there could potentially be a maximum threshold of properly paired TCRs on surface that can result in increased T cell function. Consequently, this effect might be different in lower affinity TCRs, in that a higher density of properly paired TCRs on the cell surface is needed to maximize T cell function.

One surprising finding was that T cells transduced with the disulfide bridge TCR expressed the lowest levels of properly paired HCV 1406 TCRs on the cell surface however, these T cells had an increase in tumor reactivity as shown by the median in fold changes above T cells transduced with the wild type HCV 1406 TCR, in Figure 19. Although the range of variability is large in T cells transduced with the disulfide bridge TCR, they represent the highest increase in fold changes across all the T cells transduced with a modified HCV 1406 TCR.

Since this reactivity data was normalized to reflect transduced T cells expressing properly paired HCV 1406 TCRs, it can be inferred that large amounts of IFN- γ was being released by a small amount of properly paired disulfide bridge HCV 1406 TCRs. This could be because the formation of the disulfide bond might not enhance proper chain pairing but properly paired HCV 1406 TCRs that do make it to the cell surface have an increase in stability due to this additional disulfide bond in the constant region. Thus, this increased stability on the cell surface allows the TCR to consistently function well without being degraded. In conclusion, the disulfide bridge TCR appears to not promote proper pairing at all, yet the occasional properly paired TCRs could be more stable on the cell surface via this additional covalent bond and consequently can result in an increase in T cell functionality.

Concluding Remarks

The purpose of this study was to compare the six TCR modifications by their cell surface expression in comparison to the wild type TCR as well as T cell function. By doing this we could determine if there is an optimal TCR modification that would be advantageous to use in TCR gene modified T cells for adoptive T cell therapy. This thesis hypothesized that by making modifications to the introduced HCV 1406 TCR, we can enhance proper TCR chain pairing among introduced TCRs and consequently increase T cell functionality. Our study revealed new insight on the idea that a higher level of properly paired HCV 1406

TCR cell surface expression does not always correlate to an increase in function of the T cell. Our findings indicate the T cells transduced with the leucine zipper TCR express high levels of properly paired HCV 1406 TCRs on transduced T cells but generally show a decrease in T cell functionality. This could be a result of the leucine zipper fusion proteins preventing proper signaling from occurring. When stimulated by HepG2:NS3⁺ tumor cells, generally all other T cells transduced with a modified TCR functioned better than T cells transduced with the wild type TCR despite varying levels of properly paired HCV 1406 TCRs on the cell surface. Optimal T cell function might require a low or high density of properly paired TCRs expressed on the cell surface depending upon the affinity of the TCR. Thus, it is critical to look at TCR expression and T cell function independently in the process of comparing these different modifications.

APPENDIX A:
ADDITIONAL T CELL FUNCTION FIGURES

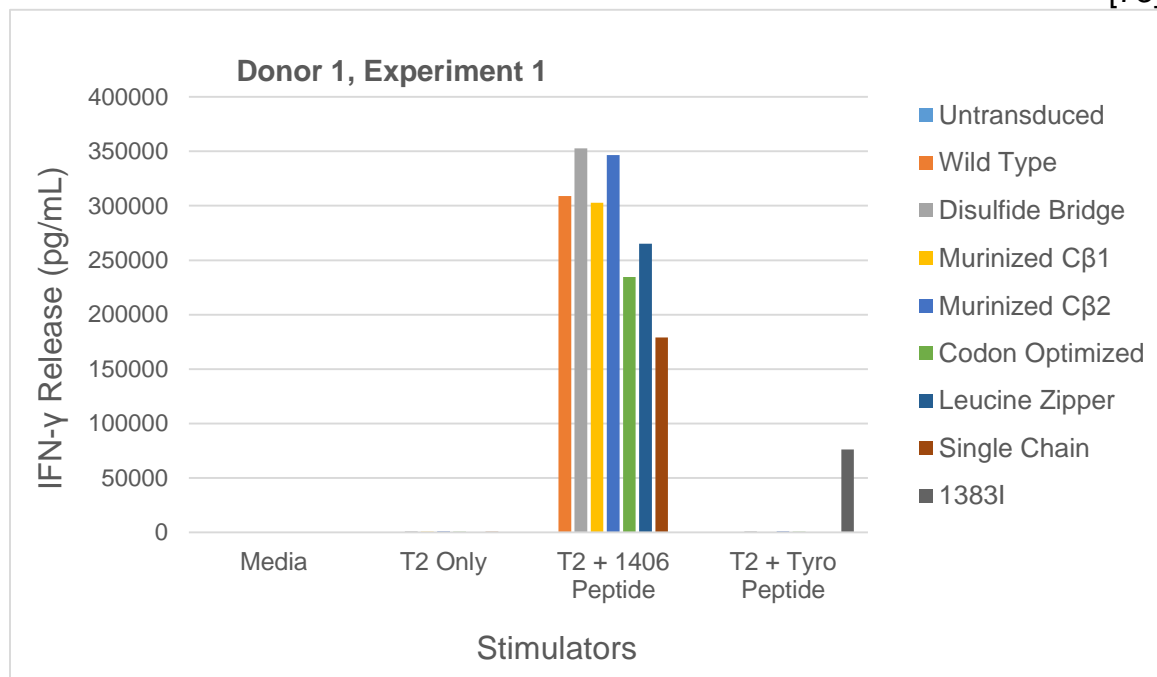


Figure 20. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 µg/mL of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN-γ release was measured by ELISA.

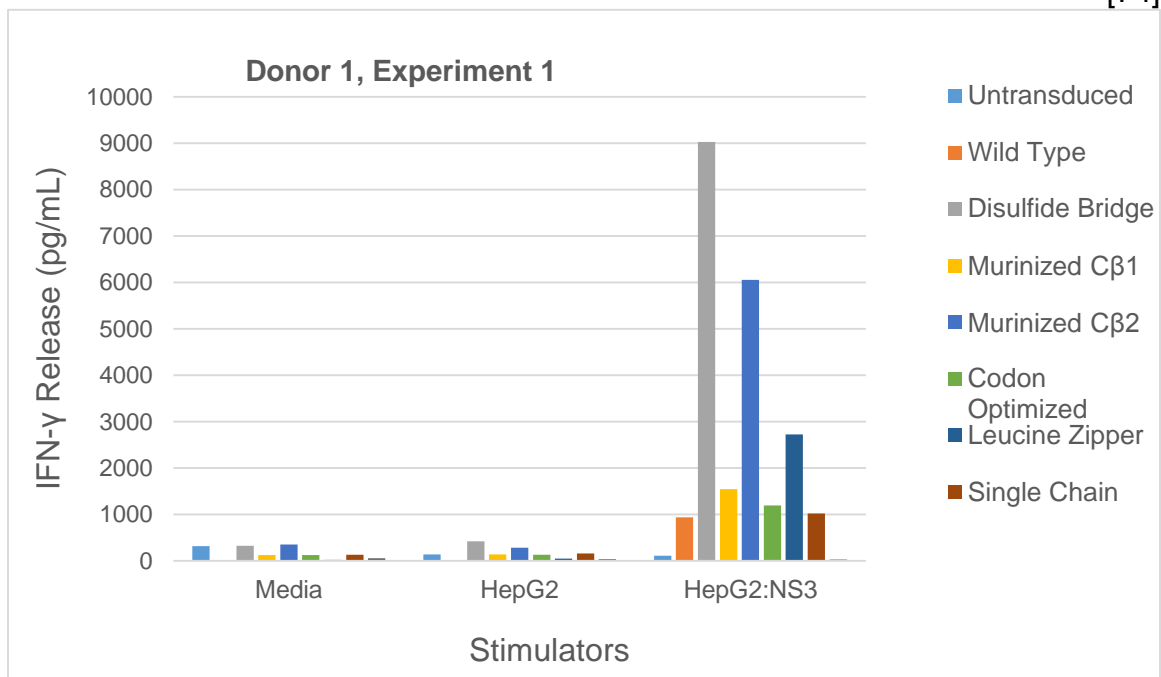


Figure 21. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN- γ release was measured by ELISA.

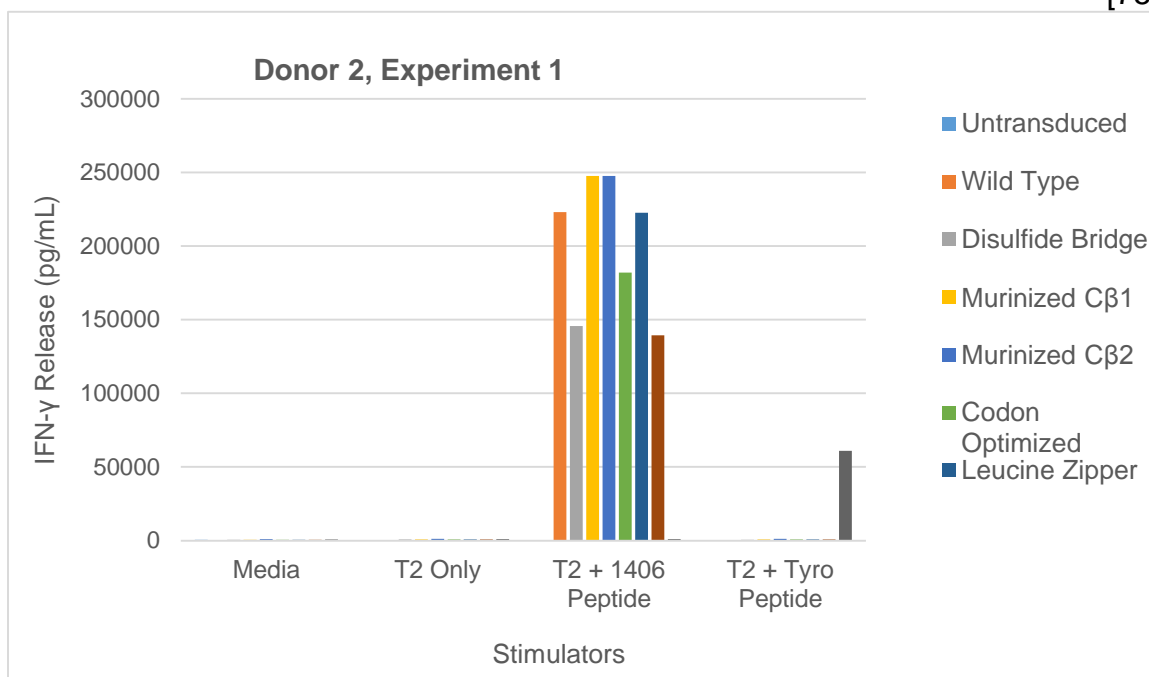


Figure 22. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 µg/mL of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN-γ release was measured by ELISA.

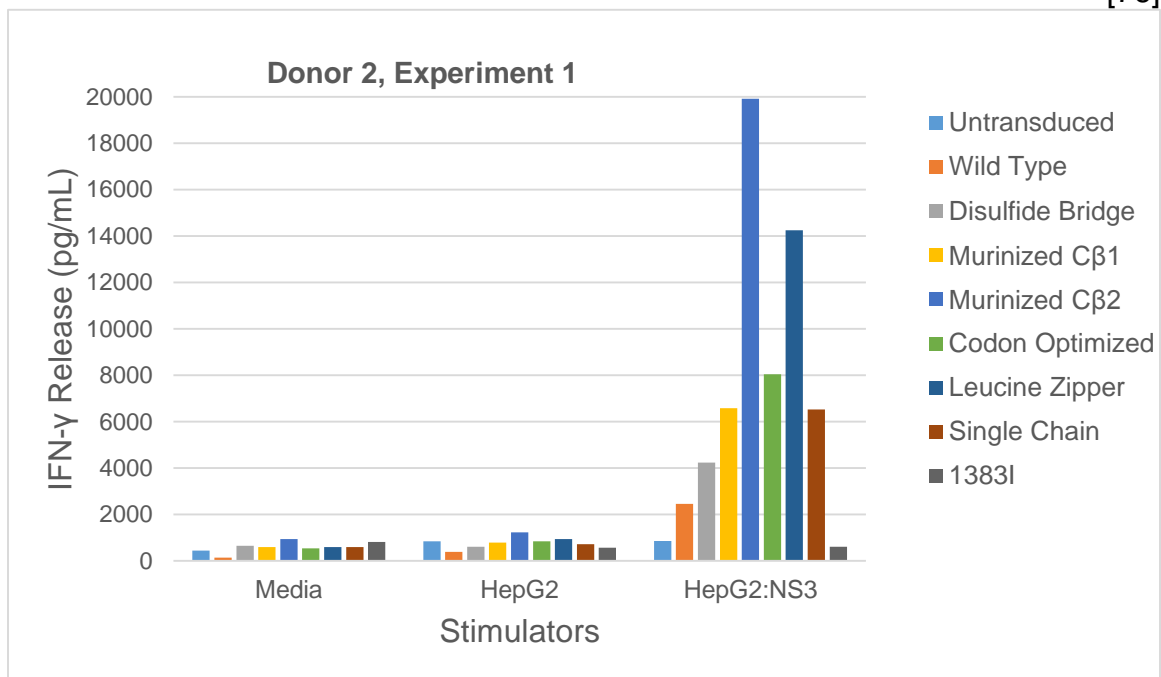


Figure 23. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN-γ release was measured by ELISA.

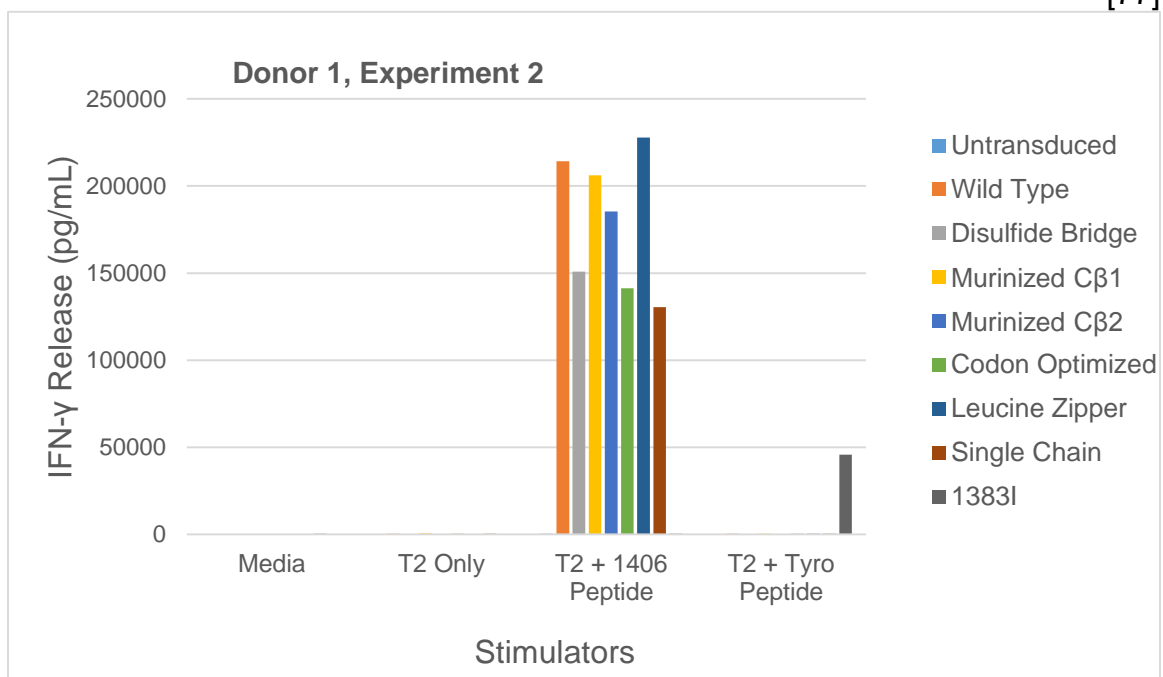


Figure 24. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 µg/mL of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN-γ release was measured by ELISA.

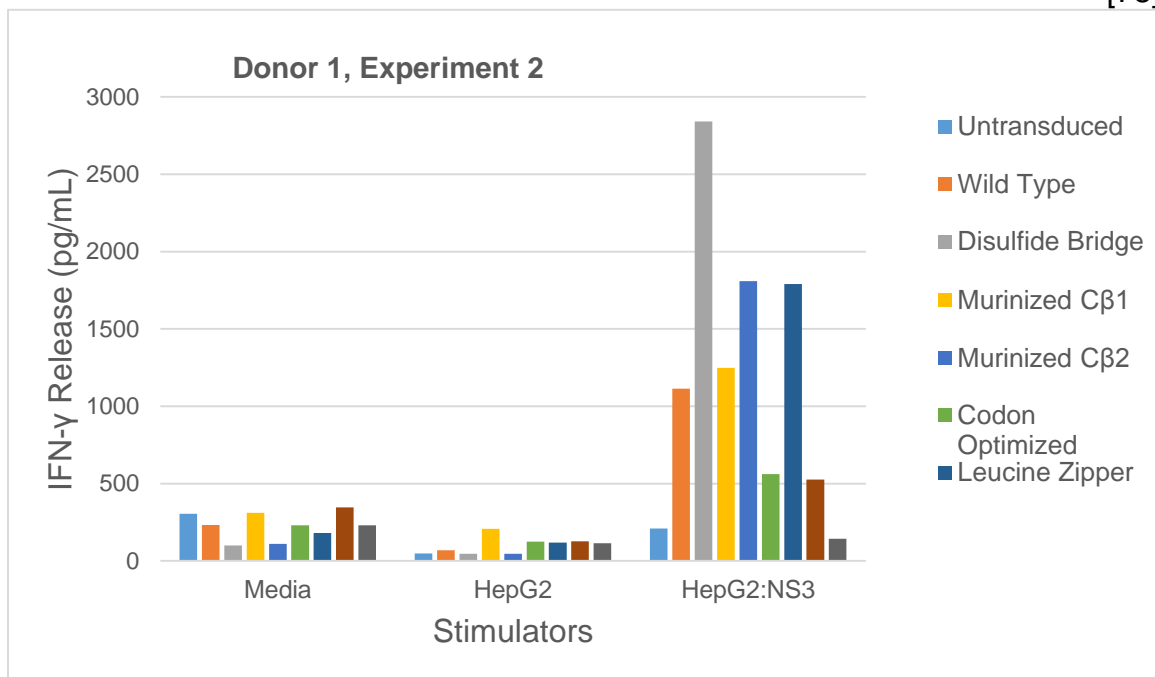


Figure 25. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN- γ release was measured by ELISA.

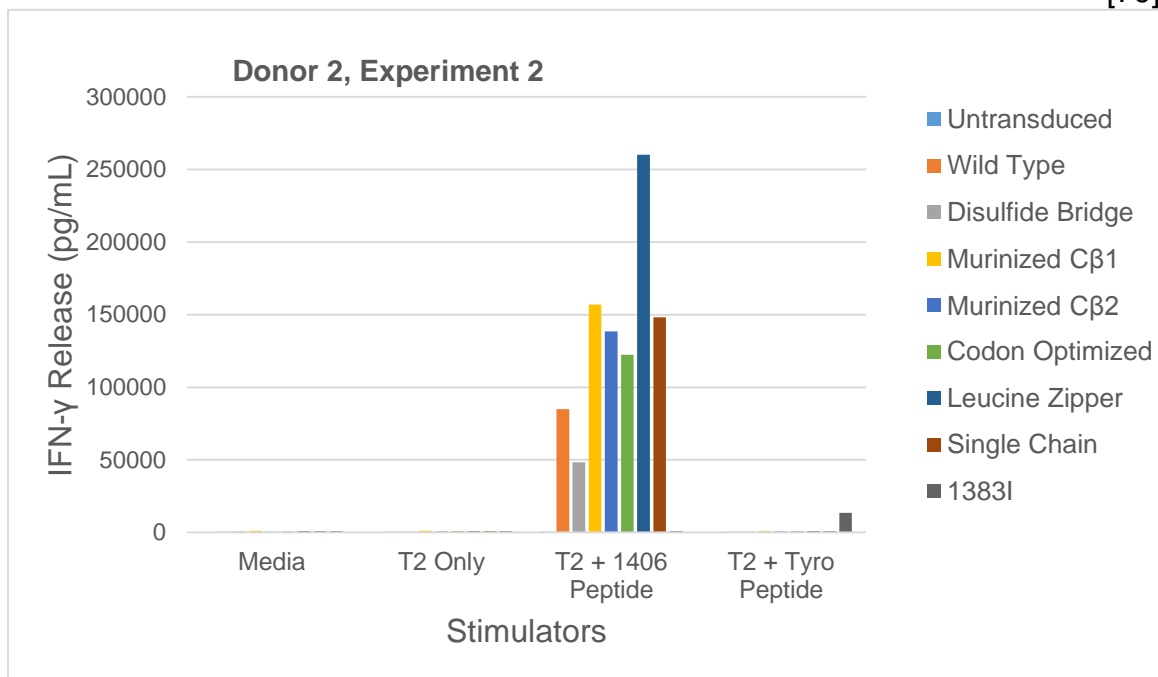


Figure 26. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 µg/mL of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN-γ release was measured by ELISA.

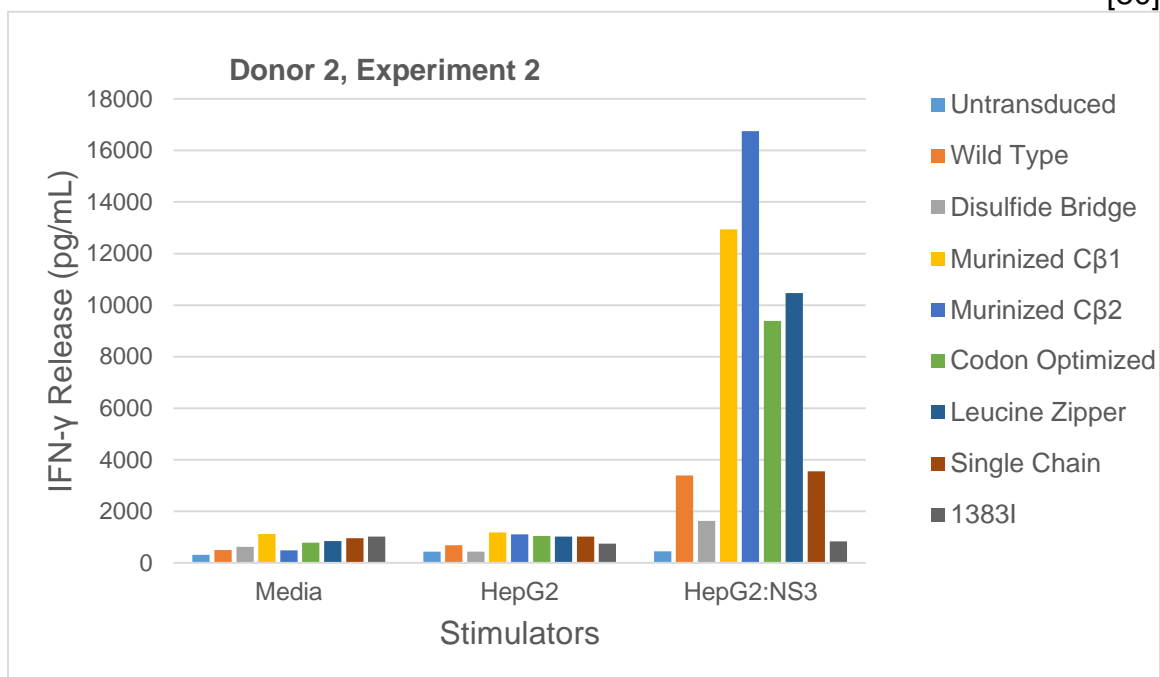


Figure 27. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN- γ release was measured by ELISA.

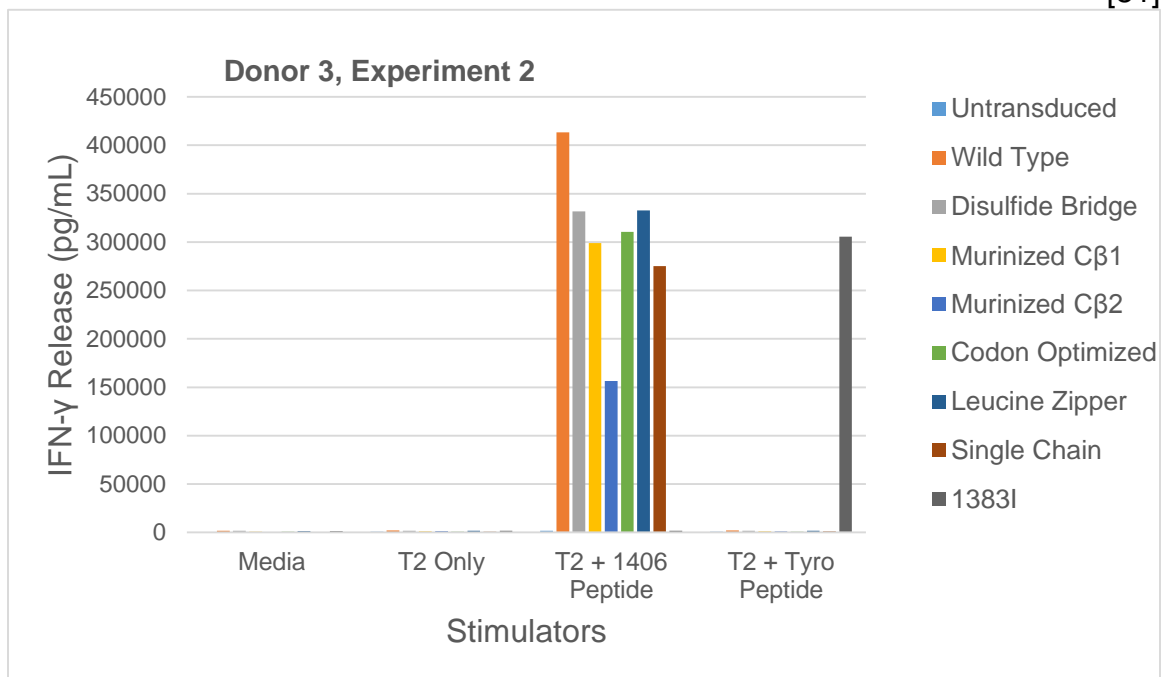


Figure 28. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 µg/mL of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN-γ release was measured by ELISA.

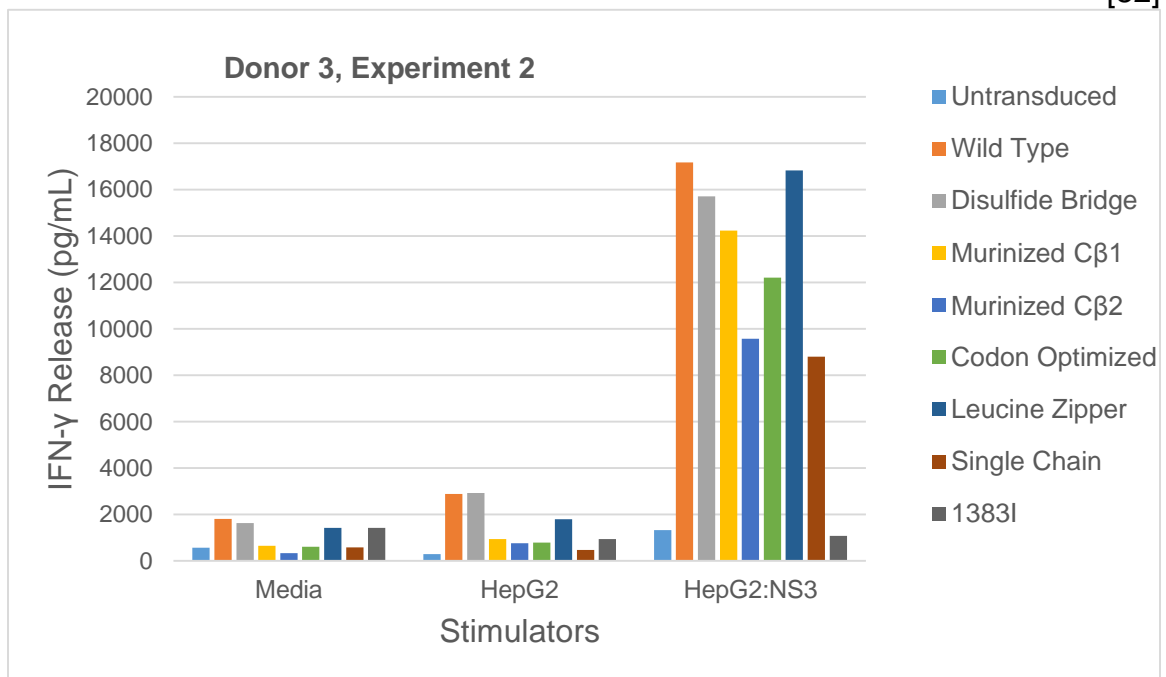


Figure 29. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN- γ release was measured by ELISA.

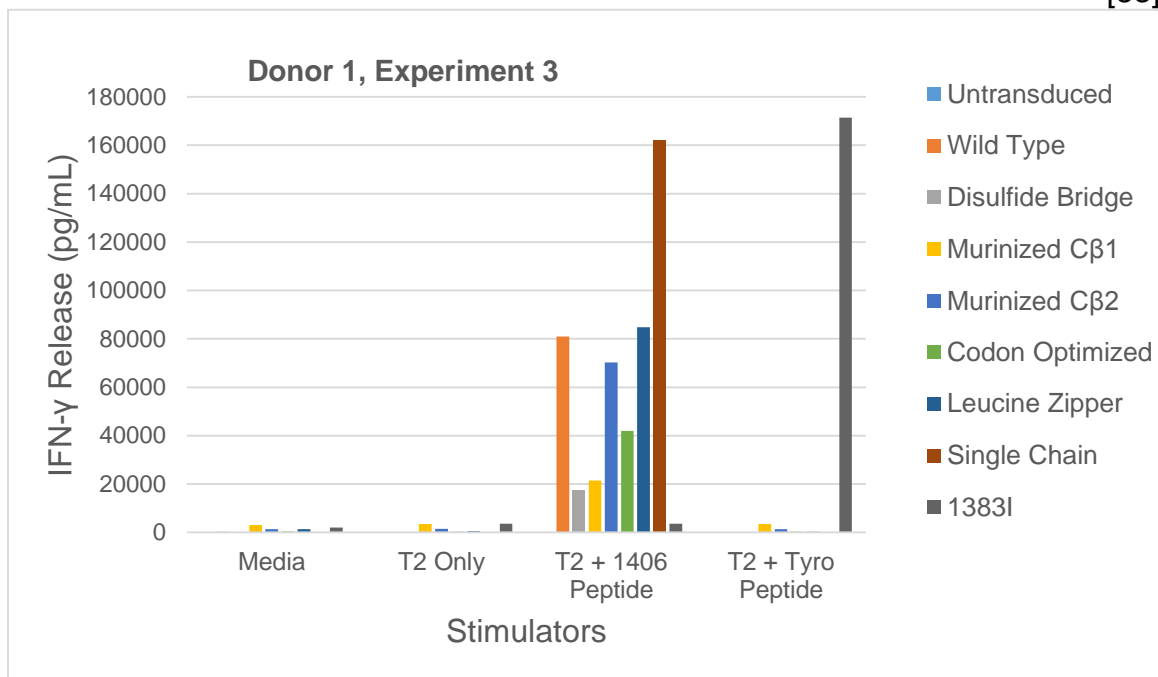


Figure 30. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 μ g/mL of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN- γ release was measured by ELISA.

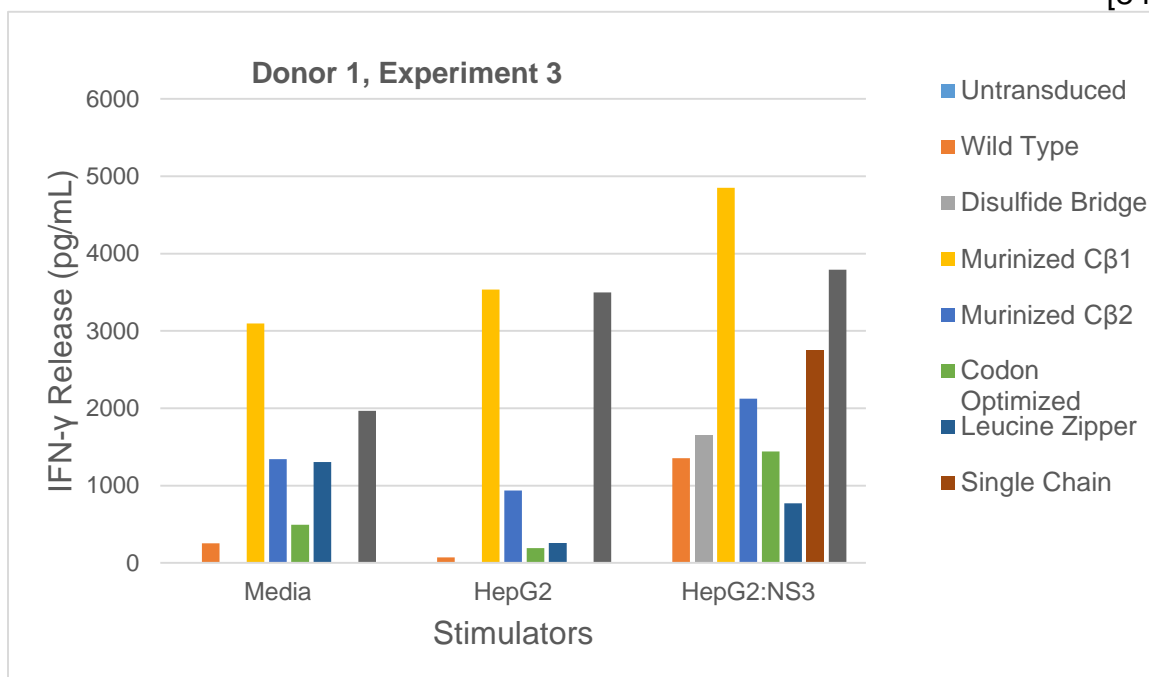


Figure 31. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN-γ release was measured by ELISA.

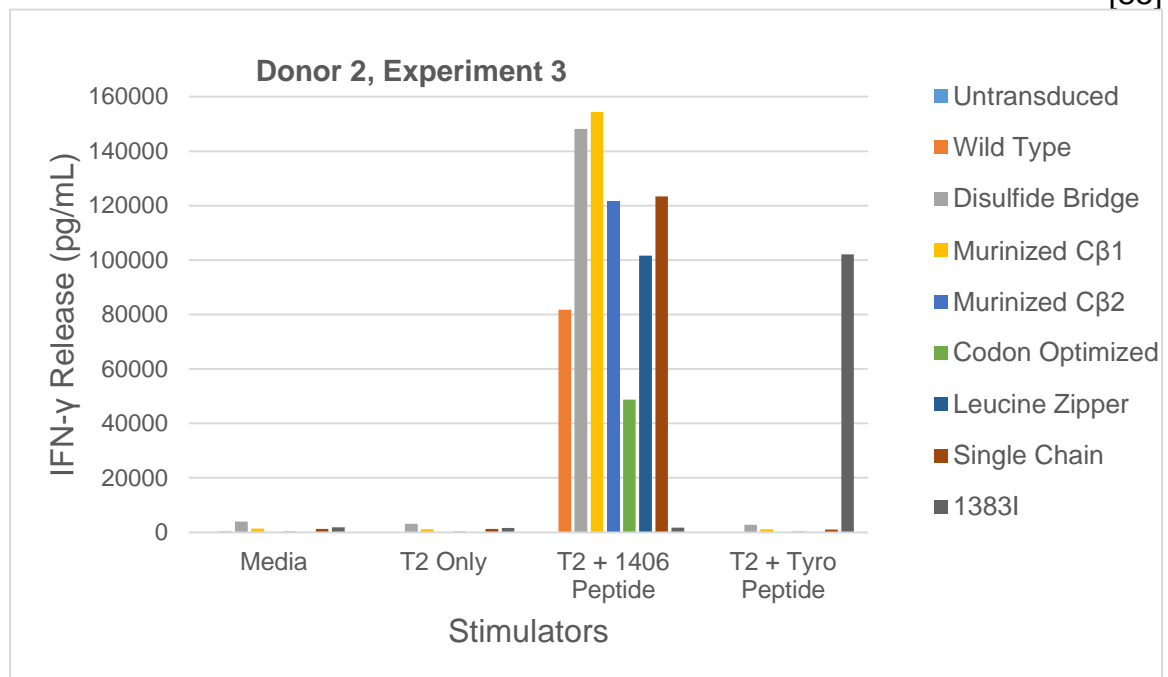


Figure 32. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 μ g/mL of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN- γ release was measured by ELISA.

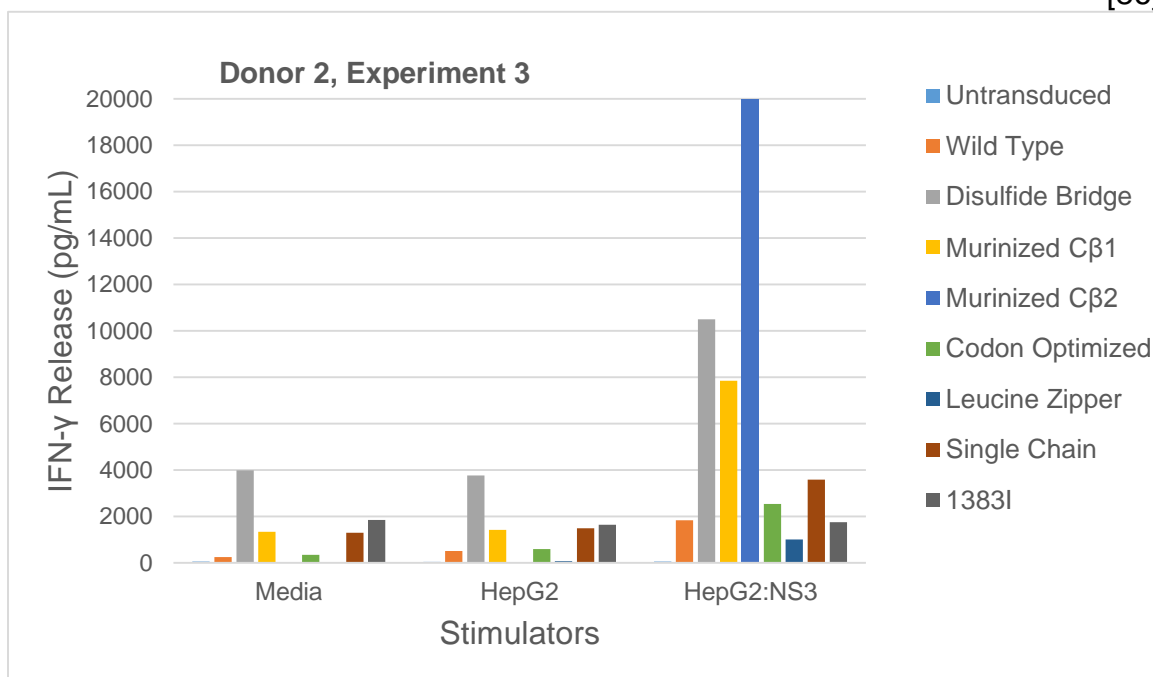


Figure 33. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN- γ release was measured by ELISA.

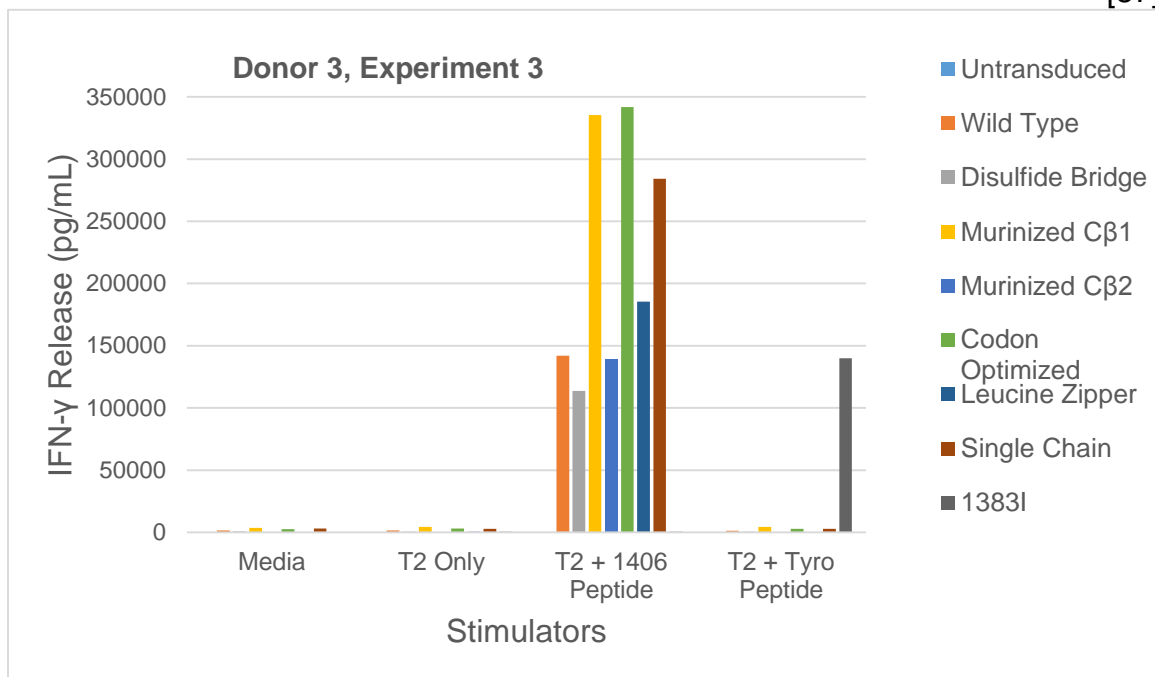


Figure 34. Comparison of peptide antigen recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

T2 cells pulsed with 10 $\mu\text{g/mL}$ of HCV NS3:1406-1415 peptide were cocultured with each TCR transduced T cell line. IFN- γ release was measured by ELISA.

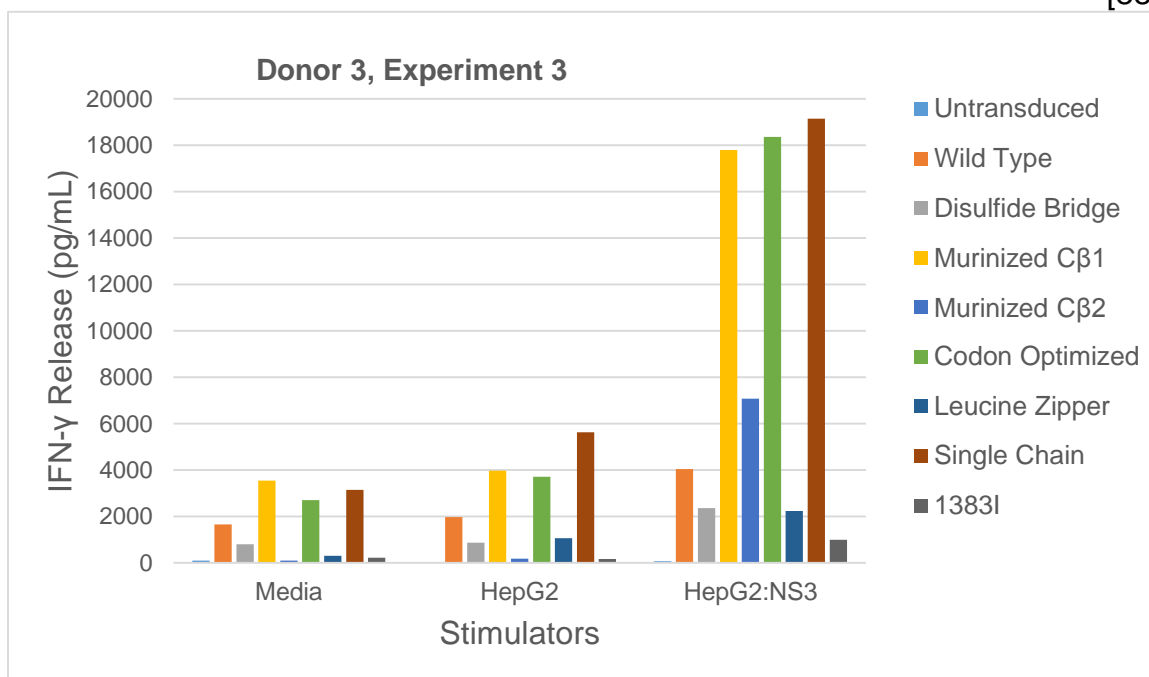


Figure 35. Comparison of tumor recognition by T cells transduced with wild type 1406 TCR or modified 1406 TCR.

HepG2 and HepG2:NS3 tumor lines were cocultured with each TCR modified transduced T cell line. IFN- γ release was measured by ELISA.

REFERENCES

1. Dudley, M.E. and S.A. Rosenberg, *Adoptive-cell-transfer therapy for the treatment of patients with cancer*. Nat Rev Cancer, 2003. **3**(9): p. 666-675.
2. Duong, C.P., et al., *Cancer immunotherapy utilizing gene-modified T cells: From the bench to the clinic*. Mol Immunol, 2015.
3. Wang, X. and M.I. Nishimura, *Chapter 18 - Genetically Engineered (T Cell Receptor) T Cells for Adoptive Therapy*, in *Gene Therapy of Cancer (Third Edition)*, E.C.L.L. Gerson, Editor. 2014, Academic Press: San Diego. p. 259-271.
4. Market, E. and F.N. Papavasiliou, *V(D)J recombination and the evolution of the adaptive immune system*. PLoS Biol, 2003. **1**(1): p. E16.
5. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision*. Nat Rev Immunol, 2002. **2**(5): p. 309-22.
6. von Boehmer, H. and H.J. Fehling, *Structure and function of the pre-T cell receptor*. Annu Rev Immunol, 1997. **15**: p. 433-52.
7. Takahama, Y., *Journey through the thymus: stromal guides for T-cell development and selection*. Nat Rev Immunol, 2006. **6**(2): p. 127-35.
8. Blum, J.S., P.A. Wearsch, and P. Cresswell, *Pathways of antigen processing*. Annu Rev Immunol, 2013. **31**: p. 443-73.
9. Wucherpfennig, K.W., et al., *Structural biology of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling*. Cold Spring Harb Perspect Biol, 2010. **2**(4): p. a005140.
10. Veillette, A., et al., *The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck*. Cell, 1988. **55**(2): p. 301-8.
11. Koretzky, G.A., *Multiple roles of CD4 and CD8 in T cell activation*. J Immunol, 2010. **185**(5): p. 2643-4.
12. Artyomov, M.N., et al., *CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery*. Proc Natl Acad Sci U S A, 2010. **107**(39): p. 16916-21.

13. Kjer-Nielsen, L., et al., *Crystal structure of the human T cell receptor CD3 epsilon gamma heterodimer complexed to the therapeutic mAb OKT3*. Proc Natl Acad Sci U S A, 2004. **101**(20): p. 7675-80.
14. Huse, M., *The T-cell-receptor signaling network*. J Cell Sci, 2009. **122**(Pt 9): p. 1269-73.
15. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T cell activation*. Annu Rev Immunol, 2009. **27**: p. 591-619.
16. Clay, T.M., et al., *Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity*. J Immunol, 1999. **163**(1): p. 507-13.
17. Rosen, H.R., et al., *Cutting edge: identification of hepatitis C virus-specific CD8+ T cells restricted by donor HLA alleles following liver transplantation*. J Immunol, 2004. **173**(9): p. 5355-9.
18. Callender, G.G., et al., *Identification of a hepatitis C virus-reactive T cell receptor that does not require CD8 for target cell recognition*. Hepatology, 2006. **43**(5): p. 973-81.
19. van Loenen, M.M., et al., *Mixed T cell receptor dimers harbor potentially harmful neoreactivity*. Proc Natl Acad Sci U S A, 2010. **107**(24): p. 10972-7.
20. Bendle, G.M., et al., *Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy*. Nat Med, 2010. **16**(5): p. 565-70, 1p following 570.
21. Kuball, J., et al., *Facilitating matched pairing and expression of TCR chains introduced into human T cells*. Blood, 2007. **109**(6): p. 2331-8.
22. Cohen, C.J., et al., *Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond*. Cancer Res, 2007. **67**(8): p. 3898-903.
23. Cohen, C.J., et al., *Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability*. Cancer Res, 2006. **66**(17): p. 8878-86.
24. Sommermeyer, D. and W. Uckert, *Minimal amino acid exchange in human TCR constant regions fosters improved function of TCR gene-modified T cells*. J Immunol, 2010. **184**(11): p. 6223-31.

25. Scholten, K.B., et al., *Codon modification of T cell receptors allows enhanced functional expression in transgenic human T cells*. Clin Immunol, 2006. **119**(2): p. 135-45.
26. Willcox, B.E., et al., *Production of soluble alphabeta T-cell receptor heterodimers suitable for biophysical analysis of ligand binding*. Protein Sci, 1999. **8**(11): p. 2418-23.
27. Chang, H.C., et al., *A general method for facilitating heterodimeric pairing between two proteins: application to expression of alpha and beta T-cell receptor extracellular segments*. Proc Natl Acad Sci U S A, 1994. **91**(24): p. 11408-12.
28. O'Shea, E.K., et al., *Preferential heterodimer formation by isolated leucine zippers from fos and jun*. Science, 1989. **245**(4918): p. 646-8.
29. Chung, S., et al., *Functional three-domain single-chain T-cell receptors*. Proc Natl Acad Sci U S A, 1994. **91**(26): p. 12654-8.
30. Voss, R.H., et al., *Coexpression of the T-cell receptor constant alpha domain triggers tumor reactivity of single-chain TCR-transduced human T cells*. Blood, 2010. **115**(25): p. 5154-63.
31. Kuball, J., et al., *Increasing functional avidity of TCR-redirectioned T cells by removing defined N-glycosylation sites in the TCR constant domain*. J Exp Med, 2009. **206**(2): p. 463-75.
32. Cole, D.J., et al., *Characterization of the functional specificity of a cloned T-cell receptor heterodimer recognizing the MART-1 melanoma antigen*. Cancer Res, 1995. **55**(4): p. 748-52.
33. Hart, D.P., et al., *Retroviral transfer of a dominant TCR prevents surface expression of a large proportion of the endogenous TCR repertoire in human T cells*. Gene Ther, 2008. **15**(8): p. 625-31.
34. Roszkowski, J.J., et al., *CD8-independent tumor cell recognition is a property of the T cell receptor and not the T cell*. J Immunol, 2003. **170**(5): p. 2582-9.
35. Norell, H., et al., *CD34-based enrichment of genetically engineered human T cells for clinical use results in dramatically enhanced tumor targeting*. Cancer Immunol Immunother, 2010. **59**(6): p. 851-62.

VITA

Kendra Foley was born on November 29, 1990 and raised in Libertyville, IL. She attended Indiana University in Bloomington, Indiana where she earned a Bachelor of Science degree in Biology with minors in Chemistry and Public Health in 2013.

After graduation, Kendra joined the Master's program in Microbiology and Immunology at Loyola University Chicago. She joined the lab of Dr. Michael Nishimura and studied an approach to minimize TCR chain mispairing and to enhance functional expression in TCR gene modified T cells to be used for adoptive cell transfer. After completion of her Master's degree, Kendra will pursue a Ph.D. in the lab in of Michael Nishimura expanding on her Master's thesis project.